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INSECT DEVELOPMENT

IV. EXTERNAL MORPHOLOGY OF GRASSHOPPER EMBRYOS OF KNOWN AGE AND WITH A KNOWN TEMPERATURE HISTORY

ELEANOR H. SLIFER¹

Department of Zoölogy, University of Iowa

ONE TEXT FIGURE AND FOUR HELIOTYPE PLATES (TWENTY-FOUR FIGURES)

AUTHOR'S ABSTRACT

Eggs of *Melanoplus differentialis*, a grasshopper, secured immediately after they had been laid, were placed in a water bath kept at 25°C. and allowed to develop for definite periods. After fixation, the embryos were dissected out of the eggs and drawn. Large numbers of eggs were examined in order to determine the degree of variation under specific conditions. For the stages following revolution, eggs were used in which 'hibernation' had been prevented by exposure to low temperatures.

From the practical standpoint, the accumulation of data concerning the grasshopper family is undoubtedly of primary importance. Uvarov, in the preface to his handbook on "Locusts and grasshoppers" ('28), emphasizes the grave nature of the 'locust problem.' According to him:

In all continents without exception and in many different countries, the interrelations of man with these insects have assumed the character of a continuous struggle, that has fluctuated in intensity, for the very existence of agriculture, and it has not been one in which man has always been victorious.

Uvarov is interested, first of all, in methods for the control of these insects, but throughout the volume he repeatedly deplores the meagerness of the information which we possess concerning them and urges strongly the wider use of the Acrididae as objects for study in university and other laboratories.

We entomologists [he says] need not insist on some special lines of investigation of our insects in research laboratories; let specialists approach the subject with their own problems and their own methods;

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let them study the anatomy, histology, chromosomes, variability, chemistry of pigments, metabolism—anything they want; we shall be able to extract from all such material what is necessary for our special purposes, while all the rest will be as valuable for science generally, as if it was gained from other subjects.

Grasshoppers, in the past, have proved themselves more than once an admirable material for biological investigation. Our present knowledge of chromosome behavior and its relation to problems of heredity is due very largely to the brilliant studies of McClung and his followers on orthopteran spermatogenesis. More recently, physiologists have begun to appreciate the value of the grasshopper for experimental purposes and we find papers on hibernation, water content, digestion, respiration, fat metabolism, etc., in which these animals, or their eggs, have served as material for research.

At this and other laboratories the eggs of various species of *Acerididae* have been found particularly suitable for studies in developmental and experimental physiology. Since in some grasshopper eggs growth is continuous from the time of laying until hatching, while in others an extended embryonic diapause occurs during which morphological changes are at a standstill, the importance of a knowledge of the state of development of the embryo under a given set of conditions soon became apparent. With this in mind the present study was begun, and it is hoped that the results will serve as an index or scale of comparison to which future work, especially that of a physiological nature, may be referred.

MATERIALS AND METHODS

Among the grasshopper eggs so far examined, those of *Melanoplus differentialis* have been found unusually valuable (Bodine, '25, '29). These, when kept at 25°C., develop for about three weeks. At this point an embryonic diapause sets in which may last for many months. Growth, for unknown reasons, finally recommences, revolution of the embryo occurs, and the completely formed animal hatches about two weeks later. If, however, such eggs shortly after being laid are

exposed to low temperatures, the diapause is prevented and, on return to a suitable high temperature, development proceeds without interruption to the end.

Material for the earlier stages of the present study was secured by keeping *Melanoplus differentialis* females, together with a few males, in cages supplied with boxes containing damp sand. When a female was found laying, she was watched carefully, and as soon as oviposition had been completed the egg pod was removed, labeled, and transferred to a deep jar suspended in a water-bath kept at 25°C. After remaining in the water-bath for exactly one week—or any other desired period—the eggs were removed, a bit of the shell clipped from the anterior end of each, and all placed in Bouin's solution. The eggs were left in the fixative for twenty-four hours or longer, then dissected under a binocular microscope, and the embryos isolated for drawing. In certain cases Bouin's solution which had been heated to the point where the first bubbles appeared in the liquid was used as a fixing agent. Whole eggs so treated were found, upon cooling, to be in excellent condition for immediate dissection. For the stages following revolution, or blastokinesis, eggs were used which had been allowed to remain buried in sand in outdoor cages from the time they were laid in September until January. These were brought inside, some fixed at once, and the rest placed in a water-bath at 25°C. A number of these were removed at the end of each twenty-four-hour interval and fixed. These eggs—series J of a previous paper (Slifer, '30)—when brought indoors had reached a stage of development corresponding to that of those kept twenty days at 25°C. from the time of laying. Hatching, in this case, occurred seventeen days later. The entire period of incubation may thus be said to have occupied the equivalent of thirty-seven days at 25°C. This is all the more interesting in view of the fact that occasionally *Melanoplus differentialis* eggs fail to 'hibernate' and, when kept at 25°C., hatch about the thirty-eighth day.

RESULTS

The majority of eggs of this species incubated at a definite temperature for a definite period after being laid will be found to have reached the same stage of development. A

TABLE 1

POD	DAYS AT 25°C.	DAYS BEHIND										DAYS AHEAD		
		10	9	8	7	6	5	4	3	2	1	0	1	2
1	5											31	3	
2	6										6	53	4	
3	7											71		
4	7										1	35		
5	8										16	58		
6	8								3		25	37	16	
7	9								1		6	75	5	
8	9							1				5	20	
9	9							4	14		1			
10	10								3		4	49		
11	10								2		5	71		
12	11						1		7		16	58		
13	11										2	38	12	
14	11							2		3	13	45	6	
15	12							1		5	26	86	6	
16	13								1	5	8	58	12	
17	14										1	33	15	
18	15							1		1	8	63	3	
19	15									1	17	78	9	2
20	16							5	7	14	11	27	3	
21	16	2	6	1		13	8	3	7	4	10	21	2	1
22	17					1	3	1	6	14	8	18	2	
23	17		1		3	2		1	1	7	3	19	33	14
24	18						4	12	9		4	38	16	
25	18						2		4	4	12	65	28	
26	19							1	3	2	10	36		
27	19							1			17			
28	19							1		4	7	43		
29	20						1	1	1	1	3	63		
30	20					1			6	20	43			
31	21			1						10		17		

certain percentage, however, will lag behind or, more rarely, be in advance of the rest, and in order to determine just how great this variation might be, large numbers of eggs with known histories were fixed and the embryos examined. The results may be seen in table 1. The eggs from a single pod

were used in each instance. Thus in one pod kept fifteen days at 25°C. sixty-three eggs had reached the stage shown in figure 11, three were at the sixteen-day stage, and ten were from one to four days behind.

Occasionally figures were secured which failed to agree with those from earlier trials. That this was not due to experimental error is indicated by the results obtained with pods 8 and 9 (table 1). Both of these pods were laid at the same time, placed side by side in the same water-bath, and fixed within a few moments of each other nine days later. Such exceptions, fortunately, are not of frequent occurrence. In contrast to this, pods 13 and 14 should be compared. These, too, were laid within a half hour of each other and kept side by side in the same water-bath until fixed exactly eleven days later. Their agreement with each other and with a third pod (12) laid at a different period is obviously satisfactory.

If a number of embryos which have reached the same stage of morphological development are examined, some will be found which are above and some which are below the average in size. This is not altogether surprising, for the eggs themselves vary greatly—some newly laid eggs weighing twice as much as others. In order to secure quantitative data concerning this point, twenty embryos which had reached the stage of development shown in figure 10 and eighty-two which had reached the stage shown in figure 16 were selected at random, the one condition being the absence of a tendency to curvature. The length from the top of the head to the extreme tip of the abdomen was measured by means of an ocular micrometer. The results are shown in figure A. Size, it will be seen, is of little value in estimating the age of an embryo.

The *Melanoplus differentialis* embryo on the fifth day at 25°C. consists of a tiny cap of cells which lies directly beneath the micropyle area at the extreme posterior end of the egg (fig. 1). By the following day (fig. 2) the germ band has grown forward along the ventral, concave surface of the egg so that a thin, flattened 'tail' (protocormic region)—roughly

equal in length to the expanded 'head' (protocephalic region) —is present. This 'tail' during the next twenty-four hours doubles or triples its length (fig. 3). On the eighth day rudi-

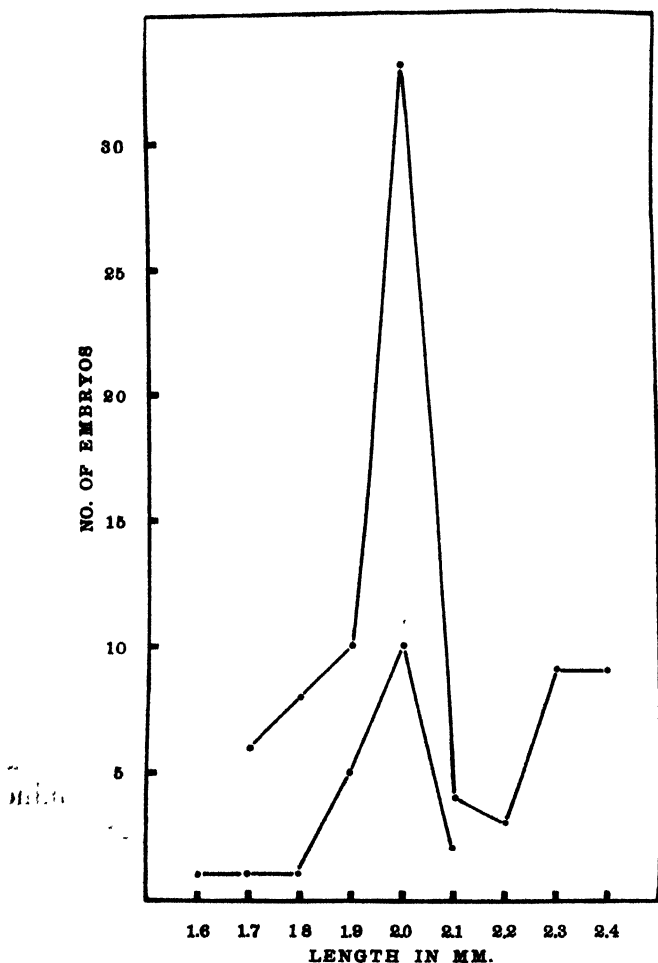


Figure A

ments of the antennae and labrum are visible (fig. 4) and the 'tail' at its upper end will be found broken into six distinct segments. The first three of these segments give rise to the mandibles, maxillae, labium, and the region surrounding

them; while the thorax with its appendages develops from the remaining three. Segmentation of the abdomen begins on the tenth day (fig. 6) and is usually completed by the eleventh (fig. 7). A day later (fig. 8) the mouth parts show constrictions in those regions where joints are to appear and the nerve cord with its ganglia can be followed, as a dim mid-ventral furrow, almost the entire length of the thorax and abdomen. In an embryo twelve days old the rudimentary legs have begun to turn inward, and by the end of the second week (fig. 10) faint constrictions can be seen where they will eventually become jointed. A notch on the posterior border of the labrum first becomes visible about this time.

The external changes occurring during the next six days consist largely of a continued differentiation of the mouth parts, legs, and abdominal segments. The embryo becomes broader, but there is very little increase in length. On the nineteenth day the ventrally situated abdominal appendages are especially prominent. These are temporary structures of problematical function and by the following day certain of them are already less distinct.

An embryo twenty days old is, judging from appearances, ready for revolution, but instead the long embryonic diapause begins and, as may be seen by comparing figure 16 with figures 17 and 18, an embryo eight days or a month older is, superficially at least, no farther advanced. If such 'hibernating' embryos are fixed, sectioned, stained, and examined microscopically, they will be found to contain few or no dividing cells. Embryos less than three weeks of age, on the other hand, display great numbers of mitotic figures (Carothers, '23; Slifer, '31).

The head of the embryo at the commencement of the diapause still lies at the caudal pole and its ventral surface extends anteriorly beneath the concave portion of the egg. All the dorsal parts are still missing, and when the embryo is viewed from the side opposite that shown in figure 16, little attracts attention save the short finger-like projections forming the stomodaeum and proctodaeum. This position

and state of development are maintained throughout the diapause. Eggs which have been 'hibernating' for a long time are frequently found with a considerable accumulation of liquid in the posterior end between the serosa and the chitinous cuticle, or 'Blastodermhaut' directly beneath the chorion.

At the close of the diapause the serosa and amnion are ruptured and the embryo moves headfirst around the lower end of the egg until the tip of its abdomen lies at the caudal pole. By turning on its long axis it then brings its ventral surface once more beneath that of the egg.² The entire process—revolution or blastokinesis, as it is called—has been followed in the living egg and found to be brought about by an active movement on the part of the embryo itself (Slifer, '32). After completing revolution, the embryo grows dorsally and anteriorly until it has enclosed the remaining mass of yolk and its head has reached the upper end of the egg. This takes place approximately eleven days before hatching.

The stages following revolution are shown in figures 21 to 24. These, as explained previously, were drawn from embryos taken from eggs exposed to low temperatures for about four months, then placed at 25°C. Embryos from eggs which have passed through a long period of 'hibernation' go through the same process and in very nearly the same time; but, since the duration of the diapause is extremely indefinite, there are practical difficulties in the way of securing the necessary stages from such material. When the diapause has been prevented by cold, nearly all eggs laid on any one day will develop together and hatch within a few hours of each other.

After the dorsal wall has closed and the embryo has reached its full size, internal morphological changes proceed rapidly, but external changes are more gradual and less obvious. The state of development of the hind femora is, perhaps, the best criterion to be used in estimating the age of such embryos. At the time of hatching the body surface as a whole is mottled with brown, the eyes are fully pigmented, the labrum is

² See Nelsen ('31) for minor variations in the usual procedure.

elaborately sculptured, the hind femora show dark 'herring-bone' markings on their external surfaces, and the leg spines as well as the mandibles are tipped with black.

The work presented here was begun at the University of Pennsylvania, continued at the Marine Biological Laboratory at Woods Hole, and completed at the University of Iowa. The author is particularly grateful for the encouragement given by Drs. C. E. McClung and J. H. Bodine. Special thanks are due, in addition, to Mr. Titus C. Evans, of the University of Iowa, for his assistance with certain of the experimental details.

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PLATES

EXPLANATION OF PLATES

All figures were drawn free-hand at a magnification of 50 and reduced one-third in reproduction. With the exception of figure 20, which is a lateral view, each embryo is shown as it appears from the ventral surface.

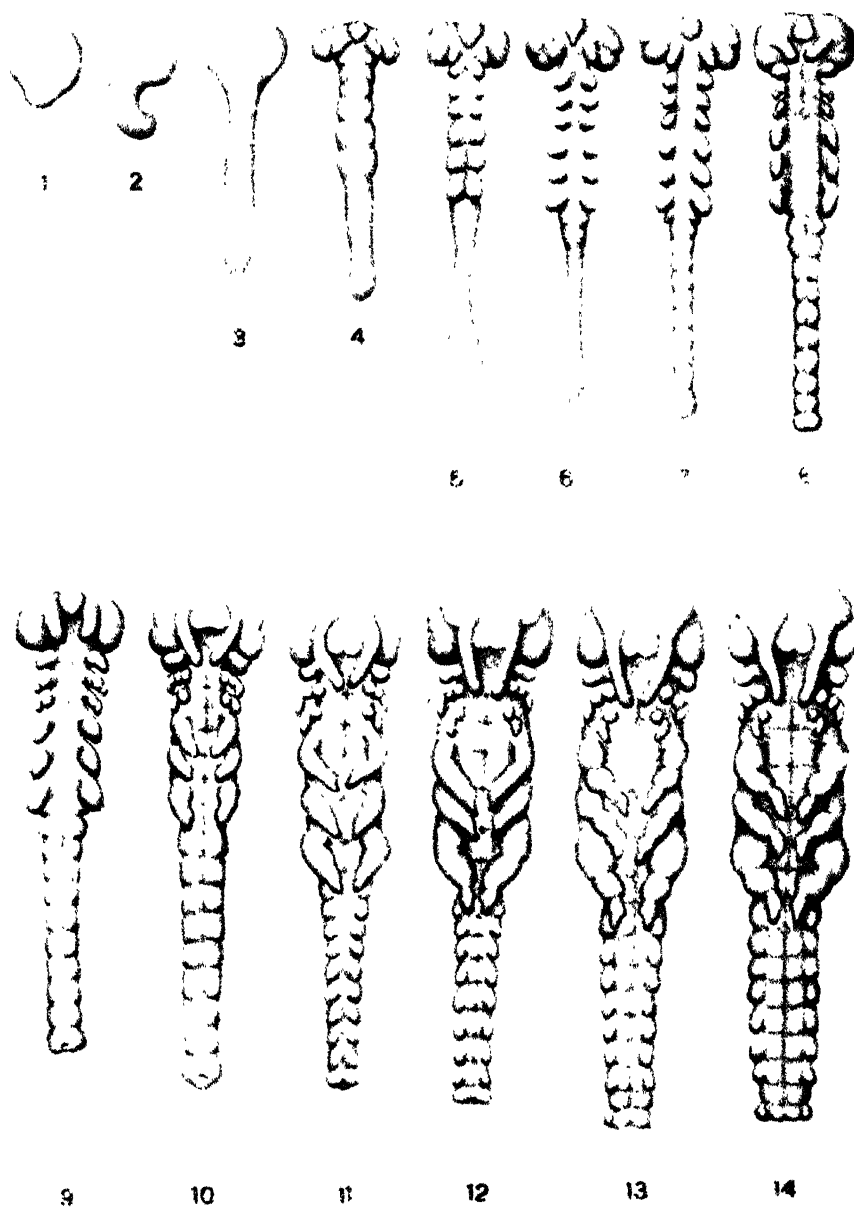


PLATE 1

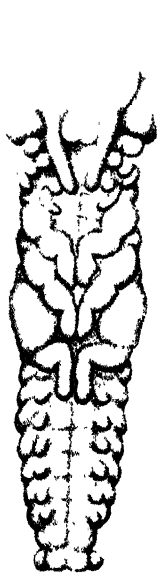
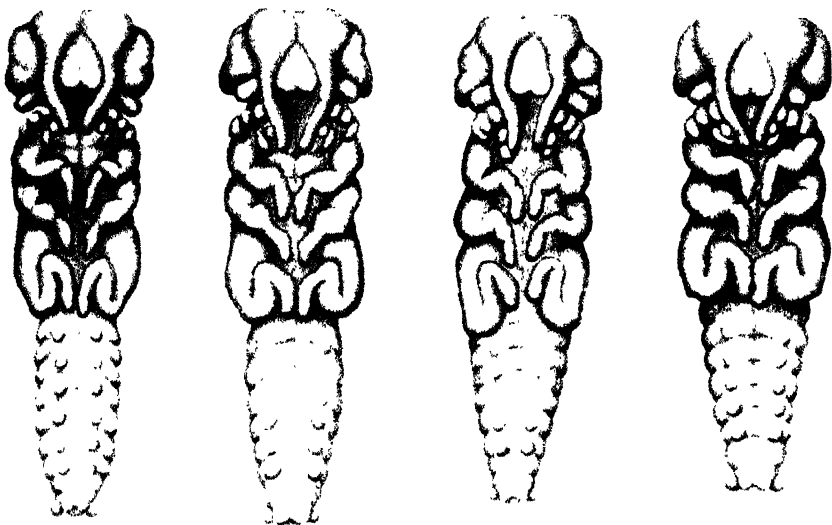
EXPLANATION OF FIGURES

- 1 *Melanoplus differentialis* embryo from egg kept five days at 25°C. from the time of laying.
- 2 Six days at 25°C. 'Tail' about same length as 'head.'
- 3 Seven days at 25°C. 'Tail' two to three times length of 'head.'
- 4 Eight days at 25°C. Rudiments of antennae and labrum visible. 'Tail' segmented at upper end.
- 5 Nine days at 25°C. Mouth parts and thoracic segments distinct.
- 6 Ten days at 25°C. First indication of abdominal segmentation.
- 7 Eleven days at 25°C. Abdominal segmentation complete.
- 8 Twelve days at 25°C. Legs just beginning to turn inward. Second and third pair of mouth parts show constrictions at site of future joints.
- 9 Thirteen days at 25°C.
- 10 Fourteen days at 25°C. Notch on posterior border of labrum distinct. Legs show constrictions at site of future joints.
- 11 Fifteen days at 25°C.
- 12 Sixteen days at 25°C.
- 13 Seventeen days at 25°C.
- 14 Eighteen days at 25°C.

PLATE 2

EXPLANATION OF FIGURES

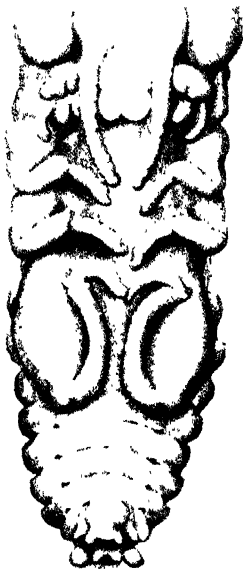
- 15 Nineteen days at 25°C. Abdominal appendages distinct. Compare position and state of development of tibiae and tarsi with that of those in figure 14.
- 16 Twenty days at 25°C.
- 17 Twenty-eight days at 25°C.
- 18 Forty-nine days at 25°C.
- 19 Series J. Fixed immediately after bringing indoors. (Seventeen days before hatching.)
- 20 Series J. Two days at 25°C. Lateral view of embryo at beginning of blastokinesis. Position in relation to yolk shown. (Fifteen days before hatching.)
- 21 Series J. Five days at 25°C. (Twelve days before hatching.)



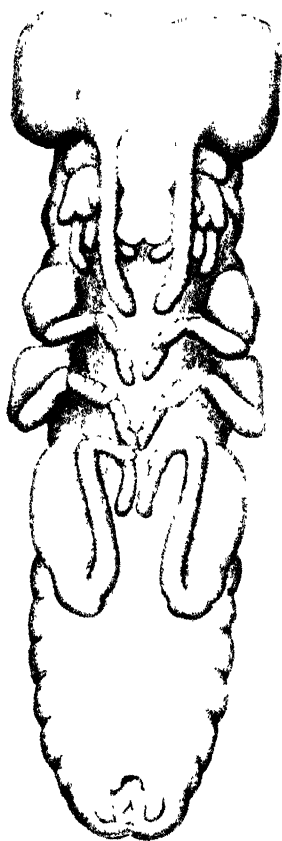
19



20



21



22



23

PLATE 3

EXPLANATION OF FIGURES

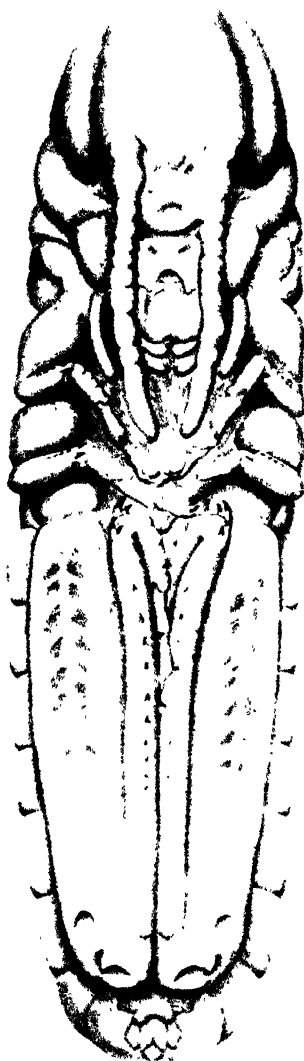
22 Series J. Six days at 25°C. (Eleven days before hatching.)

23 Series J. Eleven days at 25°C. Head of embryo at anterior end of egg and yolk completely engulfed. (Six days before hatching.)

PLATE 4

EXPLANATION OF FIGURE

24 Series J. Seventeen days at 25°C. Day of hatching.



24

THE HISTOLOGY OF POSTERIOR REGENERATION IN THE POLYCHAETE CHAETOPTERUS VARIOPEDATUS¹

G. H. FAULKNER²

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SIXTEEN FIGURES

AUTHOR'S ABSTRACT

After amputation the wound is closed by the protrusion and lateral expansion of the alimentary canal over the cut muscles and its final fusion with the epidermis laterally after about twenty-four hours. Associated with the expansion is a divergence of the two adjacent halves of the ventral nerve cord in the last remaining segment. As a result, the whole of the new regenerated lobe behind the ring of the cut epidermis is interneural, i.e., it is an enlargement of the normally small area separating the two nerve cords. Undifferentiated cells, or neuroblasts, are normally located interneurally, and during regeneration these neoblasts proliferate throughout the enlarged interneural zone of the papilla and migrate throughout the lobe. At first the neoblastic mesenchyme filling the lobe is loose and homogeneous, but as the cells settle down against the outer wall they replace the old intestinal cells and assume the characters of new epidermis. Internally, the mass acquires paired cavities, while the cells surrounding the cavities differentiate into muscle, peritoneum, etc. Thus one type of cell gives rise to the whole of the regenerated papilla. These cells are also the mother cells of the gametocytes.

Phenomena of growth may be very profitably studied among annelids. The particular type of growth associated with metameric segmentation is very plastic, and within the phylum—even among the Polychaeta alone—there are various modifications and complications superimposed on the normal life cycle, growth-maturity-reproduction.

This paper is a further item contributing to the demonstration of the correlation existing between the phases of the polychaete life cycle, and supporting the indication that there probably exists a simple and uniform histological mechanism underlying the superficial diversity.

¹The material for this work was collected at the Woods Hole Marine Biological Laboratory during August, 1929; the subsequent work was completed in the Zoological Department, University of Chicago, Illinois, and the Anatomy Department, University of California, Berkeley, California. The author wishes to thank Dr. F. R. Lillie for his assistance.

²Commonwealth Fellow.

For convenience, the phases of growth activity and of reproduction may be considered under several headings; these, however, are arbitrary, and the lines of division between them are not rigid or real.

1. Normal growth in length at the hind end of abdomen.
2. Regeneration following injury.
3. Regenerative growth on stock and bud associated with transverse fission.
4. Production of collateral buds.
5. Somatic changes associated with sexual maturity.
6. Development of germ cells.
7. Regeneration of germ cells.

Chaetopterus collected during the month of August are sexually mature and not growing; hence the first item on the list can be discussed in the historical section only. However, in a metamerically segmented animal, growth in length is always possible theoretically, and even when the full normal size is reached, the growing point persists in a resting condition and may at any time resume activity. In such a fully grown individual, the hindmost segments are small and arrested in their development, and although there are differences between such stages of arrested growth and the corresponding stages during active proliferation, yet the non-growing individual indicates to some extent the method of production of segments. Moreover, everything that is seen in the resting stages must be included in an explanation of the method of growth, and hence must be considered under the first heading.

Such continuous growth is possible, because the growth entails merely the addition of more somites and the multiplication of organs, but does not necessitate increase in size, or any other change, in the previously formed organs and tissues.

RESTING GROWING POINT

The hind end of the abdomen tapers gradually and terminates posterior to the last parapodium in a median lobe ventral to the anus—the pygidium or anal papilla.

Internally the coelomic cavities become progressively smaller, and the last one is associated with the last parapodium. The anal papilla has no coelom, but is entirely posterior to the last coelomic cavity. It is composed of a thick fibrous epithelium, in continuity with the epidermis on one hand and with the intestinal epithelium on the other.

Surrounding the intestine is a large peri-enteric sinus, occupied ventrally by a mesenchymatous tissue as in figures 4, 8, and 9, as described by Probst ('29). It is attached to the peritoneum and separated from the intestinal epithelium. In it cell outlines are indistinct, but nuclei are clear. Slender muscle fibers cross it in all directions and are especially abundant in the midventral region. Here they lie obliquely and cross each other, forming a muscular band beneath the intestinal epithelium (fig. 9). The peri-intestinal muscles are in two sets, a longitudinal sheath against the basement membrane of the intestinal epithelium, and a sheath of circular fibers external to the sinus and against the splanchnic peritoneum.

The peri-enteric sinus and its mesenchymatous contents accompany the intestinal epithelium to its extremity, and hence extend to the posterior end of the body. Thus the sinus forms the postcoelomic internal cavity—i.e., the cavity of the anal papilla (fig. 12).

The ventral nerve cords lie adjacent to each other in the ventral body wall against the epidermal cells, and interrupt the main longitudinal and circular muscles. A few isolated circular fibers lie external to the cords. A supplementary series of circular or transverse fibers lies internal to the longitudinal muscles, and these fibers are abundant against and between the nerve cords, lying internally to them (figs. 14 and 15). Posteriorly the nerve cords make a semi-circular turn anteriorly and dorsally, following the curve of the epithelium at the rim of the anus where it changes from epidermal to intestinal. They are continued against the basement of the ventral intestinal epithelium for several segments, related to it in the same way as to the epidermis

ventrally. In the anal papilla some nerve fibers insert themselves among the epithelial cells and terminate there.

From the above description it is seen that in a transverse section there are two sets of transverse muscles ventrally, one at the epidermal insertion of the ventral mesentery internal to the nerve cords, and the other at the dorsal or intestinal insertion of the same mesentery.

Nephridia are omitted from this description, as they have no close relation to the subject matter of the paper.

In addition to the tissues and organs mentioned above, there are certain cells in the hindmost segments which are of the nature of an embryonic tissue, and their distribution is as follows.

As the somites become smaller at the hind end, they have relatively smaller coelomic cavities and thicker walls; the series finally ends in a solid block of cells lying in the anal papilla. This solid block in the postcoelomic region is the homologue of the hollow somites more anteriorly, and the youngest somite is more or less a transition between the two (figs. 2 and 12).

Cells of similar nature to those forming the terminal cell mass form the thick and irregular walls of the last coelomic cavities, and more anteriorly—where the coelomic walls are thin and membranous—some cells of the same type can still be found within the walls. There is, in fact, a longitudinal row of such cells in the ventral abdominal wall lying against and between the two nerve cords among the inner transverse muscle fibers, and the posterior cell mass is merely a local expansion of this thin line. The cells are neoblasts, according to the origin and significance of the term as given by Korschelt ('27, p. 299), and since the posterior cell mass described above must be considered as being homologous, it will in future be described as the terminal or posterior neoblastic anlage. It is difficult to find a better term, and actually this one has a double significance: as seen later, it is not only the source and origin of other neoblasts, but it is also the neoblastic source of all other tissues—under certain conditions.

The precise and complete distribution of neoblasts terminally is as follows. They are anatomically adjacent to the nerve cords, and accompany these along all the lines indicated above (p. 25). In the abdominal segments they are restricted to a thin line in the interval separating the two cords, while posterior to the last somite they are more numerous, and are piled several cells deep—though they still retain their position against the nerve cords. The U-shaped intestinal continuation of the nerve is accompanied by a continuation of the procession of neoblasts, and this expands to form a layer of cells spread out over the basement membrane of the intestinal cells in the subanal region (fig. 1). In the next two or three somites anterior to this, a few neoblasts are still visible against the intestinal cells, restricted to isolated groups. Accompanying the nerve fibers which enter the epithelium of the anal papilla are also a few neoblasts, especially at the anterior and posterior extremities of the papilla, at the point of transition between it and epidermis on the one hand and intestine on the other. Some remain among the fibers of the anal muscle, and accompany this laterally and even dorsally around the anus. These are more numerous during regeneration, as described later.

Topographically, just as the epidermis, anal papilla, and intestine form a continuous curved epithelium, so do the nerve cords and the accompanying row of neoblasts—all three systems maintaining the same relationships throughout.

Figure 2 shows the distribution of neoblasts immediately dorsal to the nerve cord and among the muscle fibers. They are not numerous in such a non-active growing point, but are most abundant in the region surrounding the last somite. Such horizontal sections demonstrate a lateral extension of neoblasts from the median row, inserted into each transverse dissepiment and lying there between the two layers of peritoneum. These are destined to form the gonads. In the last-formed and youngest somite they are seen, and even in the location of the septum next to be formed. On the more anterior septa they have multiplied and form a prominence

ABBREVIATIONS FOR ALL FIGURES

Arrow in figures 1 and 2 indicates position of insertion of new somites. In other figures arrow indicates midventral line. * * indicates plane of amputation, and therefore line of junction between old and new tissues, either externally or internally.

Figures 1 to 11 were drawn with the aid of a Zeiss Zeichenapparat, and all are combinations of several consecutive sections.

an., anus
bl., blastocoel
c.c.p., posterior coelomic cavity
g., gonad
g.c., germ cells
int., intestine
int.n., intestinal nerve
m.an., anal muscle
m.l., longitudinal muscle
n., neoblasts
neo.an., neoblastic anlage

n.i.n., interneural neoblasts
n.s.c., subintestinal neoblasts
n.m., neoblastic mesenchyme
p.e.m., peri-enteric mesenchyme
p.e.s., peri-enteric sinus
p.p., posterior parapodium
pyg., pygidium
s.n., new somites
v.mcs., ventral mesentery
v.n.c., ventral nerve cord

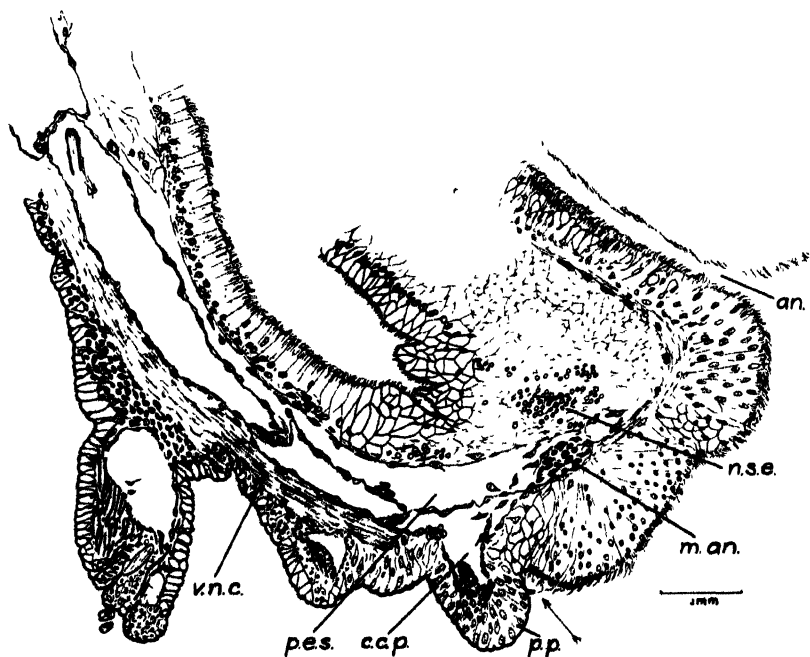


Fig. 1 Longitudinal section through ventral half of normal posterior end.

on the anterior face of the septum, but always they are covered by peritoneum.

In corresponding transverse and sagittal sections, some neoblasts are seen migrating up the ventral mesentery and into the peri-enteric sinus, lying adjacent to the splanchnic peritoneum.

These neoblasts, although they form one continuous and connected series, may be grouped for convenience of refer-

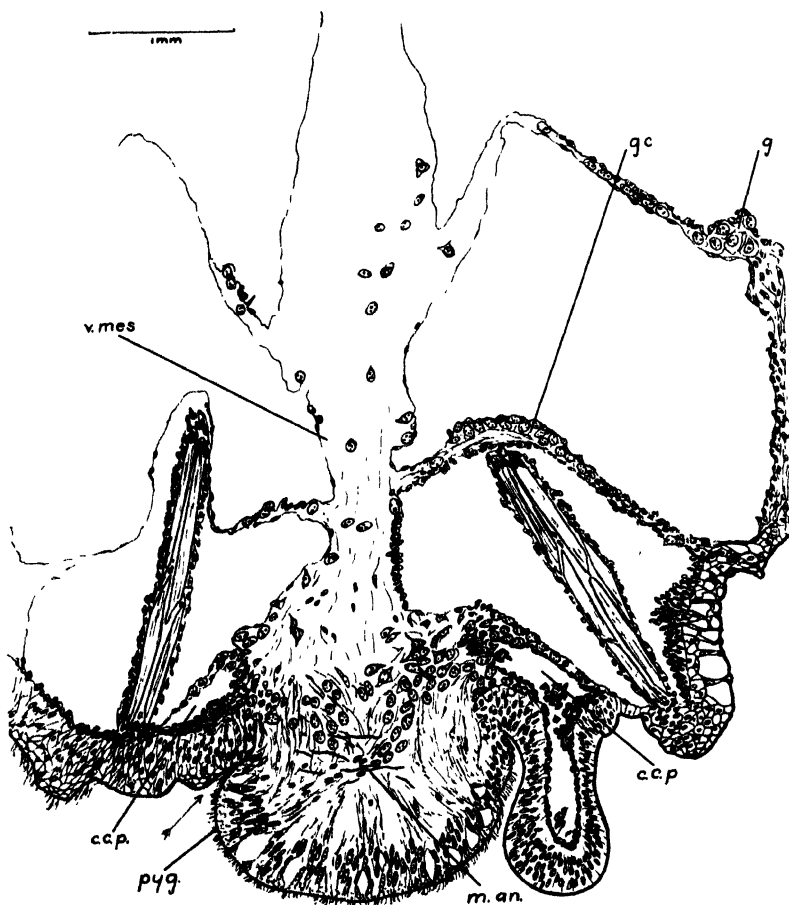


Fig. 2 Horizontal section through normal posterior end, in plane between the nerve cords and intestine.

ence according to their locations. Those in the ventral body wall will be called interneurals; those against the intestinal epithelium, subintestinals; those against the splanchnic peritoneum, splanchnics; those among the fibers of the anal muscle, laterals and dorsals; those associated with the anal papilla, pre- or postpygidials; those on the septa, the genitals.

The relations of the neoblasts to growth and production of somites, as far as can be discovered from resting growing points, are as follows. The youngest somite is found immediately anterior and lateral to the anal papilla, in the angle between it and the last parapodium (marked by arrow in figs. 1 and 2). It follows that the anal papilla and its contents are permanent structures, and that if any one point can be designated as the growing point *sensu strictu*, it is here, at the tip of the arrow in the figures. In terms of internal anatomy and neoblasts, the somite arises within the anterior end of the neoblastic anlage, within a nest of neoblasts, therefore as a schizocoelic cavity. Internally the somite is bounded by the intestine, which, as described above, is supplied with neoblasts (subintestinals), and externally, by epidermis with which neoblasts are also associated.

This last statement is difficult to establish with certainty, but from such sections as that figure in figure 2, it seems that at the tip of the arrow neoblasts are being incorporated into the epidermis, contributing to its formation (prepygidials). The part played by the subintestinals in growth of the intestine is more certain, as they are frequently seen penetrating the basement membrane and inserting themselves among the ciliated epithelial cells.

Hence, the neoblasts produce the rudiments of the internal parts of the new somite; they aid and contribute to the formation of the epidermis and intestine in the newly developing somites; they supply the neoblasts bequeathed to each new segment; and they form the rudiments of the gonad; i.e., they are the definitive germ cells.

Their behavior and activity during regeneration will be considered next. From the previous descriptions it is shown

that it is invariably true that these cells lie in the blastocoel and are always separated from the coelom by a covering of peritoneum (except in somites so young that this has not yet been formed). Moreover, one may expect to find them in any cavity which is blastocoelic in origin, and nowhere else. These relationships remain equally true during the changes associated with regeneration.

REGENERATION

Many animals when brought from the sea are regenerating their posterior ends. This process of regeneration can be induced in the laboratory by amputating the hind segments, since the animals live very satisfactorily in finger-bowls provided with a slow current of water. About twenty-five such experiments were performed, upon which the following observations are based. The time of year was August, and all the individuals used contained ripening or mature sexual organs. The cuts were made at various points on the abdomen, behind the third segment, with no apparent difference at the different levels. Moreover, in some cases a second regeneration was induced and this appeared to be no different in rate of growth from the first.

Following amputation, the cut edge of the alimentary canal curves outward, covering the cut edges of the longitudinal muscles, and in approximately twelve hours fuses with the epidermis; thus the normal anal relations are restored. The protruding circular swelling formed by the exposed surface of the alimentary canal is at first covered with a greenish brown scar, which may persist for one, two, or three days. When it is finally lost, the protrusion has been replaced by a conical outgrowth resembling a small but normal tapering hind end.

This conical outgrowth enlarges and soon shows bud-like prominences, the rudiments of the new parapodia. The papilla increases in length continuously, and new parapodia are added postêriorly.

The rate at which regeneration progresses varies slightly, but the following examples were typical.

Four days after amputation of the hind end, the conical papilla is formed—one on the fifth day measured 3.5 mm. On the sixth day three individuals are recorded as having parapodial lobes. From the eighth to the thirty-second day, the number of regenerated somites, as counted by parapodia, was as follows: 8 days, 4 somites; 9 days, 4 somites; 10 days, 2, 3, 5, 5, 6, 6 somites (six individuals); 12 days, 5 somites; 13 days, 3, 3, 5, 5 somites; 15 days, 5 and 7 somites; 17 days, 7 and 8 somites; 18 days, 7 somites; 19 days, 5 somites; 21 days, 9 somites; 32 days, 9 somites.

INTERNAL CHANGES

As described above, the intestinal epithelium is reflected over the cut surface of the longitudinal muscles and fuses with the epidermis. Thus the point of closure of the wound is not terminal, but lateral. Figures 3, 13, and 14 show the relationships at this time. The splanchnic peritoneum, being attached to the gut, is reflected with it, and unless the cut is made immediately behind a septum, comes into contact with the somatic peritoneum and fuses with it, thus closing the posterior coelomic cavity again. Moreover, the peri-enteric mesenchyme occupying the sinus between the gut and the splanchnic peritoneum is also extended with these laterally, and fills the space posterior to the last coelom. The nerve cords also diverge, and terminate at the same lateral level as the closure of the wound, as a result of which the interneural area is widely expanded posteriorly.

Differences between such a hind end and a normal growing point are as follows: there is no anal papilla; the terminal neoblast anlage is absent; the ventral interneural neoblasts are few; the subintestinal neoblasts, laterals, and dorsals are absent; the nerve cords diverge and terminate laterally at * and *, hence the interneural area is very wide.

On the other hand, essential similarity is maintained in the interrelationships of the tissues. For instance, the anus remains always open, the posterior coelomic cavity is associated with the posterior parapodium, and posterior to both

there is an internal sinusoid space—blastocoelic—but filled with a cellular mesenchymatous tissue in place of the normal neoblastic contents.

For the restoration of normal conditions the neoblasts are entirely responsible.

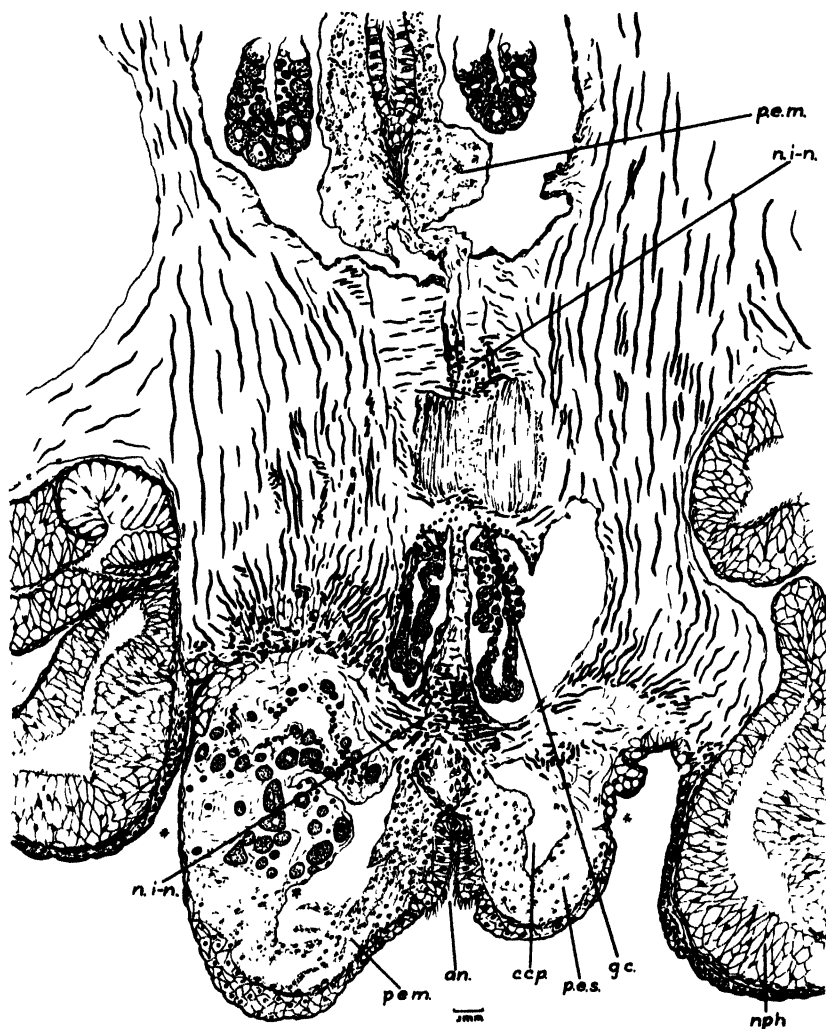


Fig. 3 Horizontal section through posterior end after twenty-four hours' regeneration; ventral to intestine.

They multiply in the interneural region and expand through the whole of the blastocoelic space, dividing further as they migrate. The terminal cavity is already more or less occupied by the peri-enteric mesenchyme, and as the neoblasts proliferate they become incorporated with this tissue and migrate through it. Figure 4 shows the close association of the two tissues, and shows them as confluent, merging one into the other. As development proceeds the loose mesenchyme cells are replaced by the more typically neoblastic cells, and it is convenient to use the term peri-enteric mesenchyme for the original tissue, and neoblastic mesenchyme for the replacing tissue present at later stages of regeneration.

The neoblasts reach the outer membrane of the hind end, and here they settle down and round themselves off, producing finally a smooth, cubical epithelium. This replaces the degenerated intestinal epithelium and forms the epidermis of the new growing point (figs. 4, 5, and 6). Further continued divisions of the ventral neoblasts result in an accumulation of cells within the papilla. These tend to congregate laterally and externally—leaving around the intestine a broad space occupied by the looser mesenchymatous tissue (fig. 9). In the subepidermal position the cells become grouped and arranged more or less in the manner of the segmental organs and tissues, and become differentiated as muscles, nephridia, etc., according to their final location. The epidermis is raised laterally in the positions of the new parapodia. The somites at first are hollow spaces, arising within the cellular mass, and the first two or three are formed synchronously. Behind the last somite, the neoblastic mesenchyme remains as a solid cell mass, the new neoblast anlage. In this way the normal relations are restored and subsequent segments are formed in regular sequence.

Details will now be given of the successive stages of regeneration as seen in serial sections.

Eight and one-half hours after amputation, the wound is still not closed, i.e., the intestinal and epidermal epithelia have not yet fused; hence the last coelomic cavity and the

peri-enteric sinus remain open to the exterior. Figure 13 shows a horizontal section through such an individual, and from this it is seen that, as mentioned above, the open wound

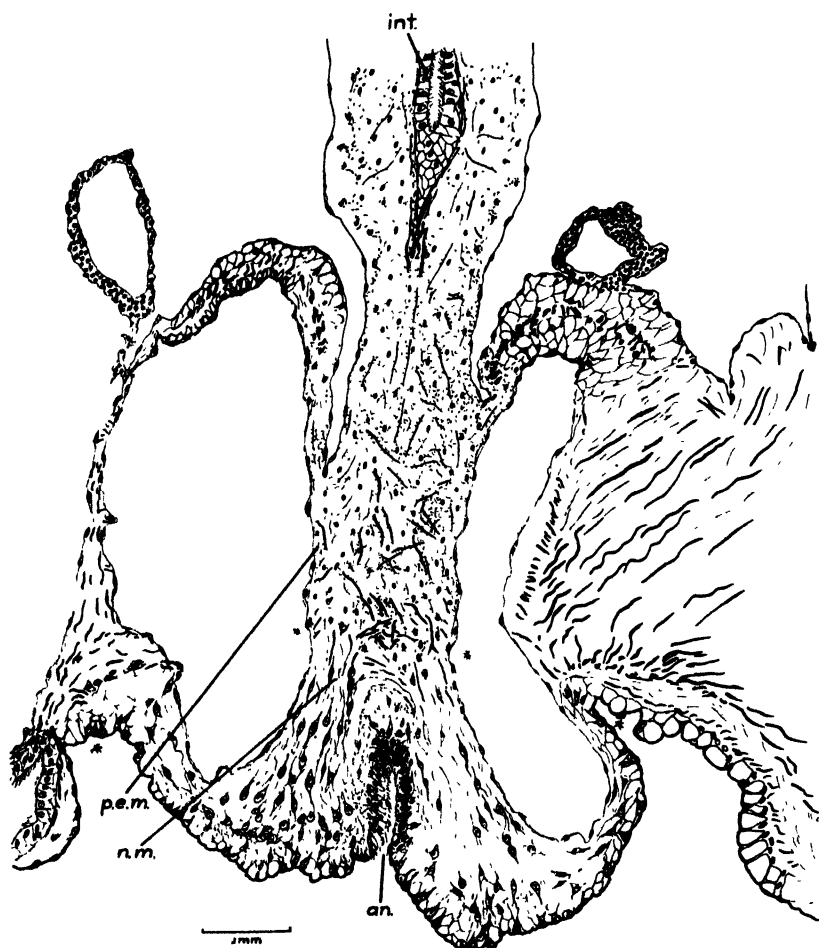
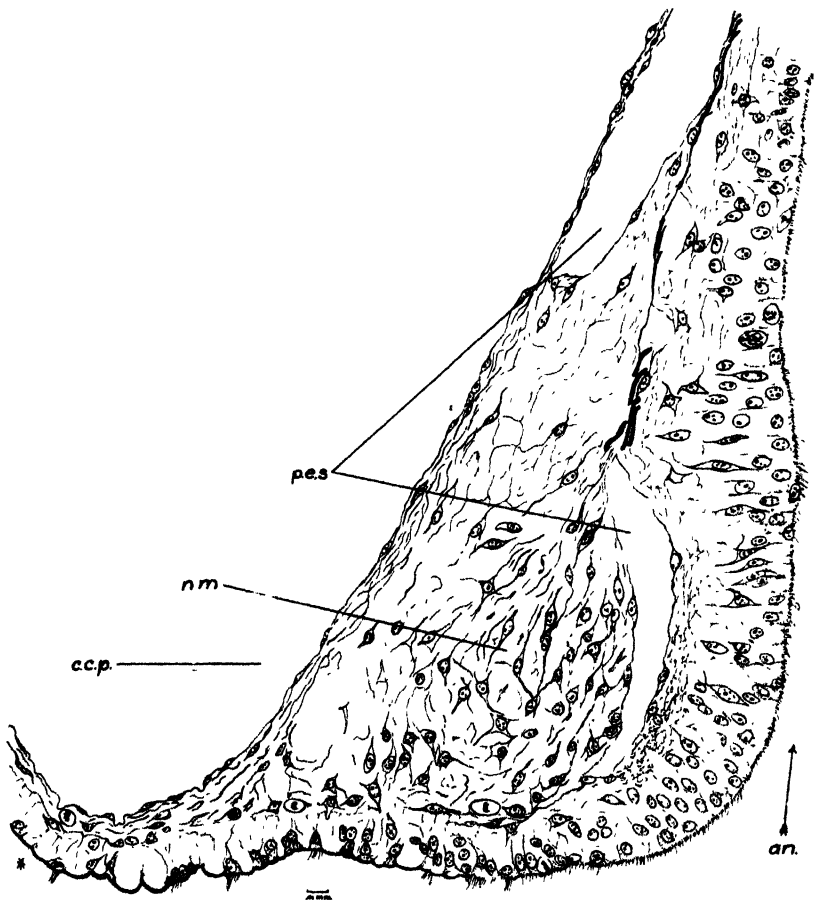


Fig. 4 Horizontal section through posterior end after forty-eight hours' regeneration; ventral to intestine.

is not terminal, but lateral. This condition results from the fact that the alimentary canal is protruded at the anus and spread over the cut ends of the longitudinal muscles; the epidermis retains its original position, hence the closure of

the wound takes place around the epidermal edge of the cut longitudinal muscles, marked ** in all figures.

The epithelium of the exposed and swollen lobe is vacuolated and more or less degenerated. The cut muscles are being sarcolized, partly by spontaneous degeneration and partly by phagocytosis. Large, shapeless, and darkly staining muscle masses are seen, and at the same time other smaller fragments are being taken up by phagocytes and



Figs. 5 and 6 Other sections from same series as figure 4, under higher magnification. Figure 5 through anus and figure 6 more ventral.

transported throughout the cavity of the terminal lobe. The possibility of phagocytosis associated with degeneration of muscles at the time of sexual maturity has been discussed by Prenant ('29), who states that in spite of previous accounts of phagocytosis, he has been unable to confirm the fact,

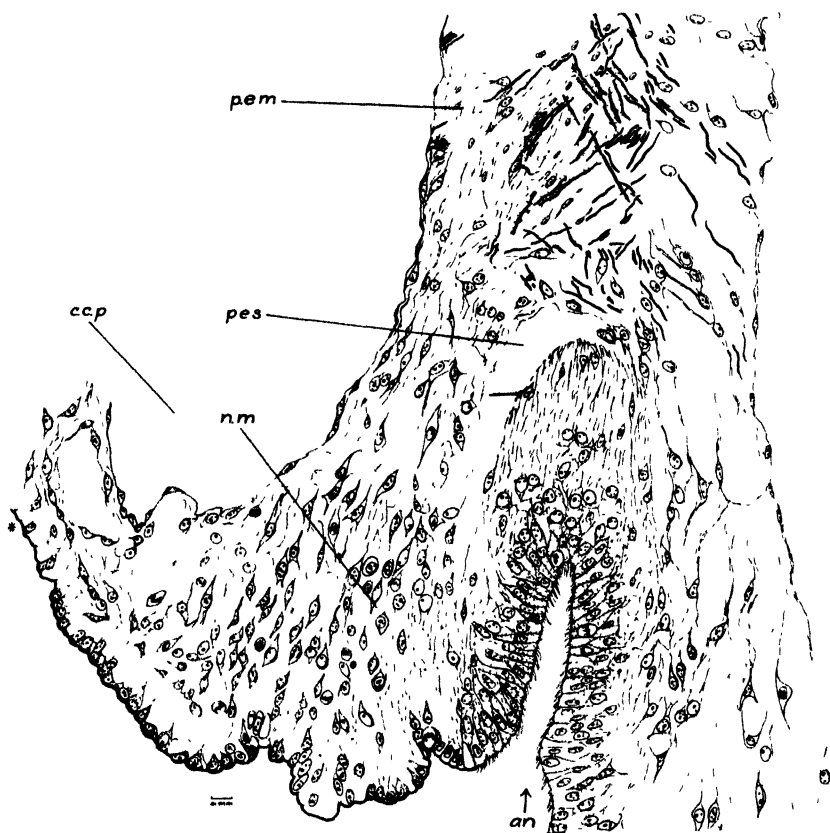


Figure 6 (see fig. 5)

though he examined many genera. However, in the case of degeneration under the precise conditions described here, the phagocytosis is evident.

The condition and distribution of the peri-enteric mesenchyme remain normal—i.e., it consists of loose tissue, the component cells of which are not distinguishable, it is

attached to the splanchnic epithelium, includes many slender muscle fibers among its cells, and coincides in its extension with the limits of the intestinal epithelium and the peri-enteric sinus; hence it forms an inner lining to the protrusion of intestinal epithelium and terminates abruptly at the plane of the closure of the wound at *.

A peculiarity of this series of sections is that the cut was made immediately behind a septum. As a result it seems that the last coelomic cavity has failed to close and has been encroached upon by the contents of the terminal blastocoel.

After twenty-four hours' regeneration (figs. 3 and 14), the wound is completely closed, though the point of closure remains evident at * and *. At this plane the nerve cords terminate, the gut epithelium is confluent with the epidermis, and the peri-enteric mesenchyme ends abruptly. As a result of the divergence of the nerve cords their cut ends are no longer transverse, but oblique (parallel arrow in fig. 15), and the interneural area is correspondingly increased in extent. The row of interneural neoblasts is divided as a result of this divergence, and one portion of the original median series accompanies each of the cords on its inner margin.

The free faces of peritoneum, splanchnic and somatic, have met and fused, so that the last coelomic cavity is again closed. The cut surface of the longitudinal muscles is marked by the presence of numerous darkly stained fragments, in all stages of degeneration and phagocytosis. The boundary of the terminal lobe is still formed by the protruded intestine, and the interior by the peri-enteric sinus and its mesenchymal contents.

The mesenchyme of this sinus is now no longer quite normal, but contains, in addition to its original loose tissue, an increased number of neoblasts ventrally. The interneural neoblasts, although they are not seen to be actively dividing, are more abundant—especially externally at the plane of the cut ends of the nerves at * and *. Moreover, some have migrated from their original location into the mesenchyme of the sinus. Many phagocytes containing muscle fragments are seen in the mesenchyme.

It has been recorded that these sections of early stages of regeneration show no cell divisions, although the neoblasts have increased in number. In later stages divisions are abundant, and it may possibly be that the few first divisions synchronize as they do in the first few embryonic cell generations. If that is so, it might be that the fixation in the few cases reported occurred during interkinesis, and that if sufficient stages were examined, divisions would be seen in some. It is seen that in young gonads of *Chaetopterus* (which are homologous with the neoblasts), the component cells do synchronize accurately in division stages.

After thirty-three hours' regeneration (fig. 15), the intestinal protrusion still forms the posterior end of the body, but now an anus is indicated. Internally the interneural neoblasts are multiplying and are migrating through the terminal sinus. Kineses are seen interneurally, especially terminally at *.

At forty-eight hours the extruded intestinal epithelium is being replaced by a conical papilla of new tissue, laid down in the manner of a tapering growing point. The original epithelium has degenerated entirely, and its place is being taken by wandering cells from the mesenchyme. In time a continuous cell layer is thus formed, and finally a basement membrane. The stages of epidermis formation are seen in figures 4, 5, and 6, all of which are drawn from the same series of sections; figures 4 and 6 are more ventral than figure 5. In all three the establishment of a definite anus is apparent, demarcating the ciliated intestinal epithelium from the epidermal.

The mesenchyme filling the cavity of the papilla has changed in character, being now formed almost entirely of neoblasts. These have more or less replaced the original peri-enteric mesenchyme, and the transition between what may be called peri-enteric and neoblastic mesenchymes is seen at the plane **.

The only sign of differentiation among the neoblasts is in the epidermal region and at this age is incomplete. After the intestinal epithelium is demarcated, the mesenchyme retreats

from the basement membrane as in figure 5, leaving a space as in a normal abdomen.

After three days' regeneration (fig. 7), the new epidermis is complete terminally, but laterally the old cells have not been entirely replaced. The anus is sharply marked and the posterior section of the alimentary canal is beginning to acquire the bulbous folded form typical of a normal abdomen.

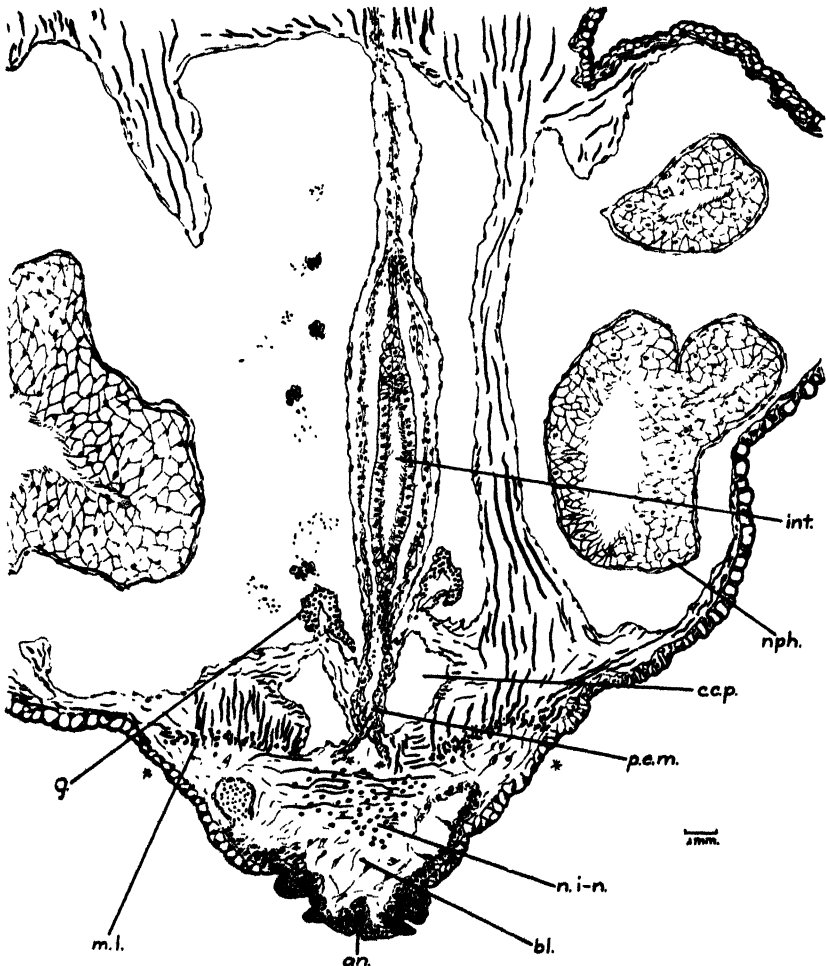


Fig. 7 Horizontal section through posterior end after three days' regeneration; ventral to intestine.

The neoblasts are now no longer uniformly distributed throughout the posterior cavity, but have retreated from the gut and are massed against the epidermis. The sudden transition from peri-enteric to neoblastic mesenchyme is seen medially at *. Three pairs of parapodia are indicated. The ventral interneural neoblasts are numerous, especially at the junction of old and new tissues, forming a broad sheet ventrally among the transverse muscles as in figure 7. Many of them are dividing (fig. 7 is more ventral than fig. 3 or 4).

At five days' regeneration, two series of sections were made, one horizontal and one sagittal. Figures 8 and 9 were drawn from the horizontal series, 8 being more ventral than 9. The sections were slightly oblique, and so asymmetrical; hence the midventral line has been marked by a longitudinal arrow in both.

The epidermis is now complete except in the ** plane dorsally. The gut is complete and normal, with the typical posterior bulbous swelling. The pygidium is indicated. Dark masses of degenerating muscles still mark the plane of the cut, and this is even further demarcated by the abrupt change in the nature of the mesenchyme. In the cavity of the papilla the neoblastic mesenchyme has retired from the median peri-enteric region for the most part, and is massed laterally. Meanwhile the deeper parts of the cavity are filled with a very loose mesenchymatous tissue. In some places this mesenchyme is still in contact with the gut epithelium, and here cells are passing from it through the basement membrane into the gut epithelium. Thus the gut is still incorporating neoblasts into itself. As the mesenchyme retreats finally from the basement membrane of the intestinal cells, it lays down the layer of longitudinal gut fibers, which, in contrast to the longitudinal fibers, are internal to the sinus. The development of these is more advanced ventrally than dorsally.

In the body wall the muscles are laid down, also the nerve cords—as seen in figure 9, both the ventral cords and the sub-intestinal termination.

Neoblasts are numerous ventrally, many of them dividing, and a terminal neoblast anlage is now distinguishable. They

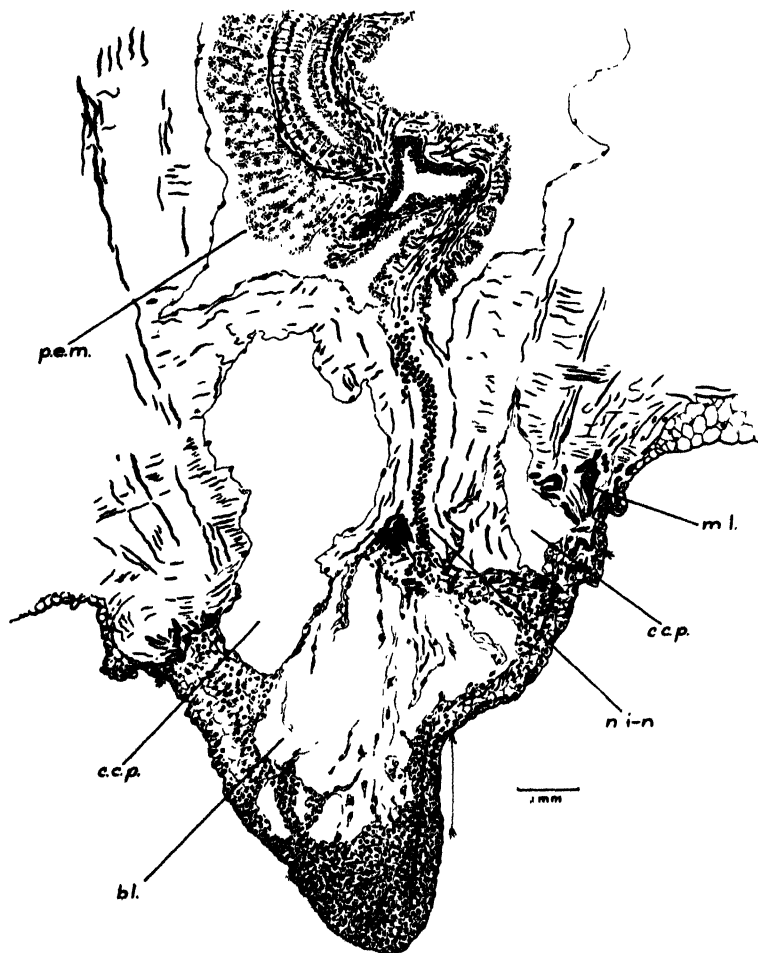


Fig. 8 Horizontal section through posterior end after five days' regeneration; through ventral body wall; section is somewhat oblique, and midventral line is marked by arrow.

are not restricted to the ventral region, but form a sheath around the intestine posteriorly, i.e., lateral and dorsal neoblasts. Cells from this sheath frequently insert themselves into the external epithelium around the rim of the anus—in

the position of the transition from epidermal to intestinal epithelium; these cells are dividing actively, thereby contributing to the growth in length of both epithelia.

Ventrally, within the masses of undifferentiated cells, cavities are forming which are the rudiments of the new somites.

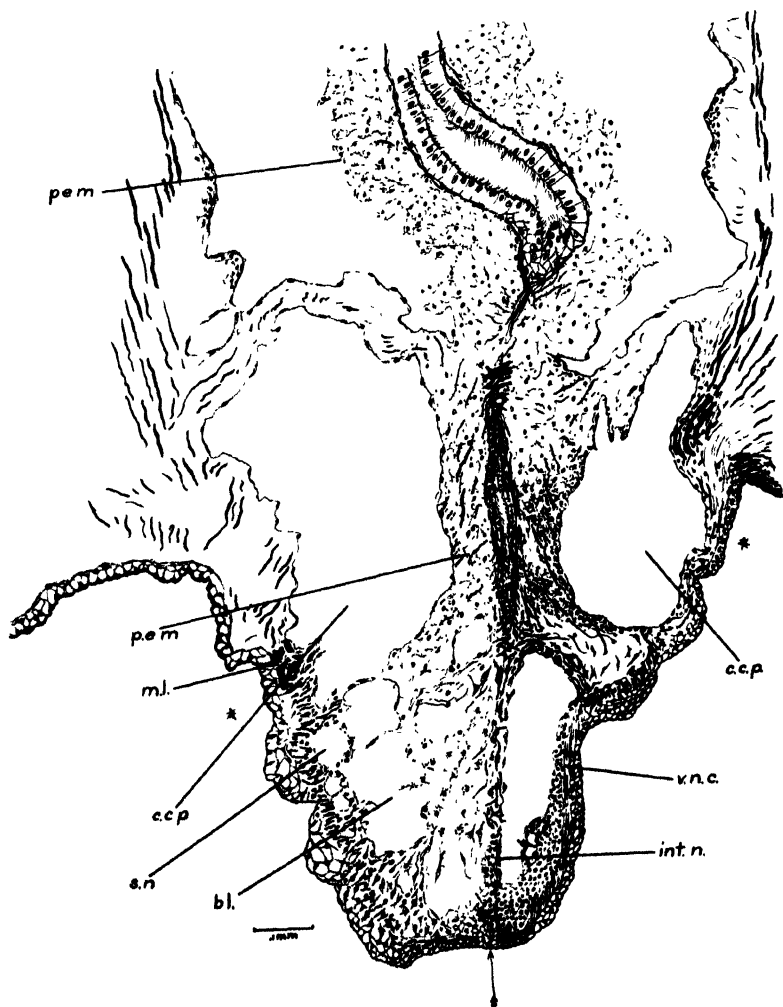


Fig. 9 From same series as figure 8, more dorsally; midventral line again marked by arrow.

They arise schizocoelically and at this time they are irregular and indefinite, and not sufficiently precisely demarcated to allow counting. The number of somites, however, is indicated by the parapodia.

At ten days the changes already begun are more advanced, but there is little new to add. Figure 10 is a transverse section taken through such an individual, posterior to the last somite. The section is slightly oblique, hence the left half is

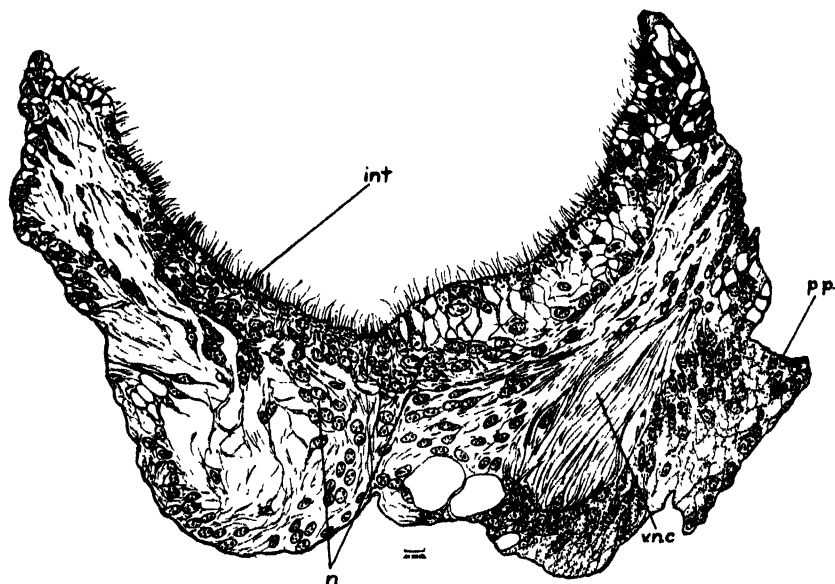


Fig. 10 Transverse section through extreme posterior end of ten days' regenerate.

more posterior than the right. The mode of formation and differentiation of the tissues is seen here. The exterior is formed by a more or less incomplete epidermis ventrally, and by the ciliated anal epithelium dorsally. The interior is filled by the termination of the nerve cords, intermingled with which are numerous neoblasts. The abundance of neoblasts medially is evident, as is also their wide distribution throughout the cavity. They are inserting themselves into the intestinal epithelium where the basement membrane is still

incomplete and are taking up positions externally where they will form epidermis. The continuity—and lack of demarcation—between all tissues is evident, the undifferentiated internal cells merging into those of the tissues.

More anteriorly, coelomic cavities lined with peritoneum are found, restricting the neoblasts to more external locations.

For later stages of regeneration several series of sections were examined, two after seventeen days' regeneration, one after nineteen days (with seven somites), and one of unknown age with ten new somites. These all agree so well that one description applies to them all equally.

The posterior somites are now well demarcated from the pygidium. Similarly the posterior neoblastic anlage is well demarcated from the hollow somites; it is particularly large, filling the whole of the blastocoelic cavity between the gut and the epidermis. Moreover, lateral and dorsal neoblastic extensions pass round the sides of the gut, and from these cells are being incorporated into the intestinal epithelium.

Figure 11 is drawn from a nineteen-day regeneration through the nerve cords, and figure 16 is a corresponding photograph.

The youngest and most posterior somite is composed of a pair of irregular spaces in the anterior end of the neoblastic mass. At first these are thick-walled, but in the older somites, as the cavity increases in size, the cells of the wall become more expanded.

At a slightly more differentiated stage, as seen more anteriorly, there is at the anterior lateral angle of the somite a thickening of the wall which later extends posteriorly, becomes hollow, and is recognizable as a nephridial tube. The oldest nephridium after the regeneration of ten somites has still the form of a simple undifferentiated and straight tube, possessing a funnel anteriorly and an external opening posteriorly.

Finally, the inner lining of cells surrounding the coelomic cavity becomes membranous and assumes the characters and relationships of a peritoneum. Ventrally the peritoneum is

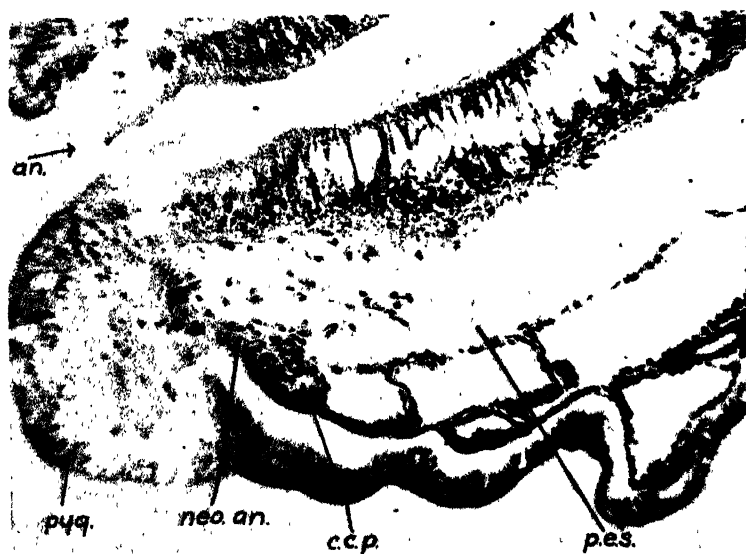
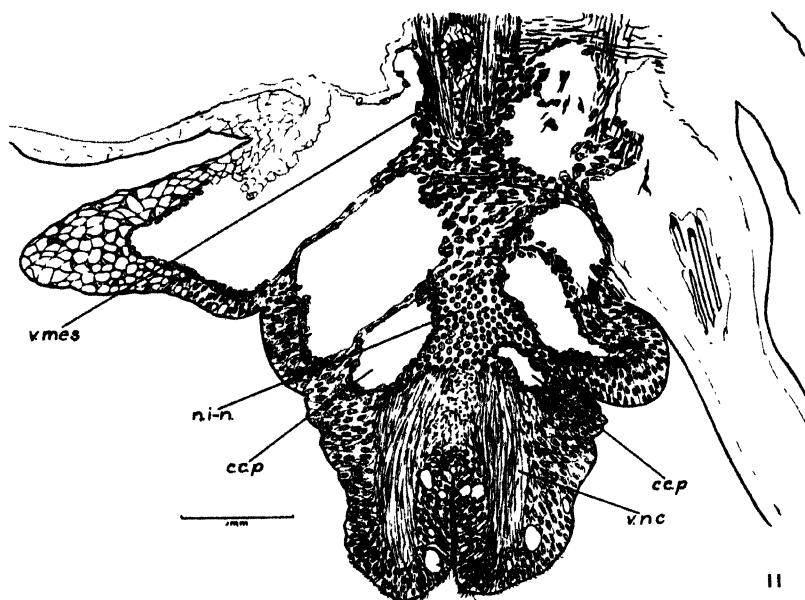


Fig. 11 Horizontal section through posterior end after nineteen days' regeneration.

Fig. 12 Sagittal section through normal posterior end, ventral half. Photomicrograph.

not completed for some time. All the regenerated somites in these series have their peritoneum still incomplete ventrally; figures 11 and 16 show the condition in the last three somites, and more anteriorly still, the walls are formed by similar



Fig. 13 Horizontal section through posterior end eight and one-half hours after amputation. The protrusion of the intestinal epithelium and the plane of closure of the wound at * are seen. Photomicrograph.

loosely packed cells medially and ventrally, though the non-membranous portion of the wall is progressively less anteriorly. While the peritoneum is still incomplete, the neoblasts retain their embryonic character and are widely distributed over the floor of the somite. In fact, they are still migrating toward—and contributing to—the centers of

growth of the various organs, nephridia, chetal sacs, etc., as seen in figure 11 in the second coelomic cavity on the right. This figure also demonstrates that the ventral longitudinal mesentery is in the more posterior somites a wide channel



Fig. 14 Horizontal section through posterior end after twenty-four hours' regeneration. The cut was oblique and the hind end is consequently curved; the midventral line is marked by the dotted black line. Photomicrograph.

solidly packed with neoblasts. Similarly, the last-formed dissepiments are thick and solid also, and become thinner and typically membranous later.

As a result of these events, the cells remaining as the recognizable, undifferentiated neoblasts are restricted to certain well-defined and constant positions in the somite; by the

completion of the peritoneum they are excluded from the coelomic cavity, and are henceforth relegated to the blastocoelic spaces—where they remain permanently. Some remain in the ventral body wall in the interneural region, and others remain in each dissepiment between the two layers of epithelium. Still others migrate up the ventral longitudinal mesentery into the peri-enteric sinus. Thus as each somite is



Fig. 15 Horizontal section through posterior end after thirty-three hours' regeneration; again the midventral line is curved and is marked by dotted line. Photomicrograph. $\times 954$.

formed, a set of neoblasts is set aside in it in the same locations as they are found permanently. These are retained throughout life as a source of undifferentiated, embryonic material.

The septal neoblasts are destined to form the gonads. From the first they produce an elevation on the anterior face of the mesentery, and by tracing these cell masses anteriorly, one sees the stages of their enlargement (figs. 1, 2, and 12): numerous cell divisions produce the sex cells of the mature

gonad, as found in more anterior somites. Figure 2 shows that the rudiments of the gonads are present in the most posterior somites. In such horizontal sections, moreover, the anatomical continuity of the germ cells and neoblasts is

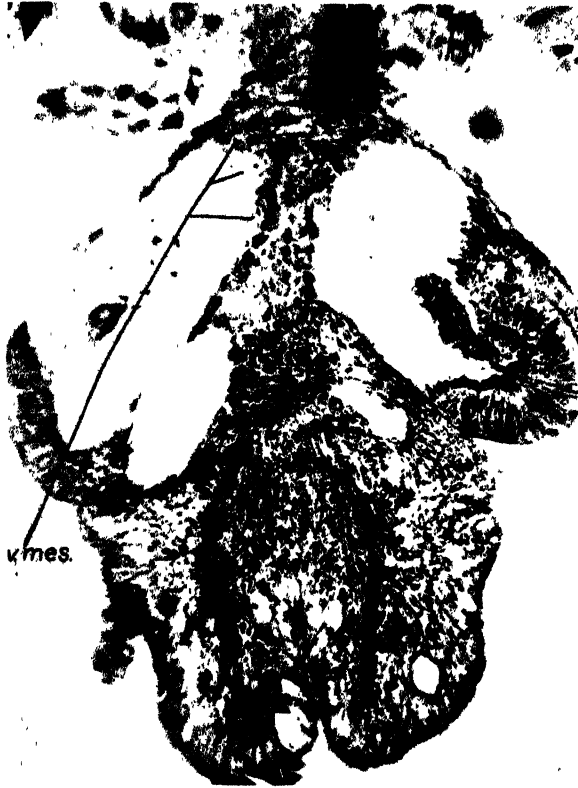


Fig. 16 Horizontal section through posterior end after nineteen days' regeneration; same series as figure 11. Photomicrograph. $\times 298\frac{1}{2}$.

always apparent in any segment in which one traces the serial sections: ventrally and mesially the two types of cells merge indistinguishably. It is clear that the germ cells do not arise from the peritoneum: they are always external to the peritoneum, and this can be seen as a thin epithelial covering over the gonads.

CONCLUSIONS

The main interest arising from this description concerns the early stages of regeneration and the means whereby an amputated abdomen with a transversely cut surface is transformed into a conical growing point. As soon as this is accomplished (approximately by the fifth day), growth continues without any special regenerative features, although it may or may not be precisely as in normal growth.

The chief difference between such a freshly cut abdomen and a growing point is the distribution of neoblasts, as described above on page 32. At the moment of amputation they are confined to the interneural region of the ventral body wall almost entirely, but as a result of the first changes they become more widely expanded. They multiply and migrate through the whole extent of the extracoelomic or blastocoelic cavities; throughout all their history they never cross a mesenteric epithelium.

Between eight and twenty-four hours of regeneration the two nerve cords diverge widely terminally, together with the associated median structures—mesentery, and peri-enteric sinus with its contents, also transverse interneural muscles. Hence, after closure of the wound, the whole of the convex posterior swelling behind the cut must be regarded as a hypertrophied interneural area. This hemispherical lobe posterior to the plane * is the seat of all future proliferation and activity, hence it follows that the outgrowth of new tissue is a production of the interneural region.

The conception of disproportionate growth of parts of an organism is familiar in other phyla of the animal kingdom.

DISCUSSION

The historical discussion centers round three main topics: first, other accounts of regeneration in polychaetes; secondly, the relationships of germ cells to somatic cells other than neoblasts, also regeneration of gonads; thirdly, the possibility of any histological similarity between the various phases of the annelidan life cycle as defined above on page 24.

The literature on Oligochaeta has recently been completely reviewed by Stephenson ('30), and will not be mentioned here, as there is nothing new to add.

I

Michel ('98) describes an undifferentiated ventral cell mass in continuity with, and arising from, the ectoderm, from which the other tissues are differentiated; he calls this embryonic tissue 'la bande germinale.'

Nusbaum ('05, '08) describes regeneration in *Nerine*, *Amphiglena*, and *Nereis diversicolor* as being essentially similar, and finds certain 'grossen charakterischen Zellen' in the neural region of the ventral body wall arising from the peritoneum and wandering through the coelom. However, in his interpretation he derives the large cells from the ectoderm, and finds that the germ cells in the new somites do not arise in situ, but wander in from the older, more anterior somites.

Iwanov ('06) agrees with Nusbaum in the case of *Nerine*, in differentiating sharply the germ cells and the tissue-producing cells, 'Urgeschlechtszellen und Keimzellen,' deriving the former from other germ cells and the latter from ectoderm. Iwanov's most recent paper is discussed later on page 54.

Okada ('28), working on syllids, gives figures which resemble those of *Chaetopterus* closely, and he emphasizes the importance of certain wandering cells which fill the terminal cavity after twenty hours. He says it is impossible to tell from where these cells come, whether from the old septa or from the body wall; if they are identical with neoblasts, they might equally well come from both places.

Langhammer ('28) describes the wandering cells active in regeneration in *Procerastea halleziana* distributed as in *Chaetopterus*; he states that they are massed ventrally above the nerve cord, though he does not suggest that this is the seat of their proliferation and origin.

Pflugfelder ('29) describes anterior regeneration in *Dio-patra amboinensis* as taking place in a way extremely similar to that described above. Regeneration is said to be accomplished mainly by the activity of certain 'Regenerations-Zellen' which he finds abundantly along the ventral nerve cord. These cells produce trophocytes, phagocytes, and lymphocytes.

Stolte ('29) finds that *Polyophthalmus pictus* is incapable of regenerating lost segments. A wound may be closed, but there is no replacement of lost tissues. There are present, however, undifferentiated and multipotential mesodermal cells apparently identical with neoblasts. It seems therefore that histologically there is no obvious explanation of the loss of the power of regeneration.

Probst ('30), in a brief preliminary account of posterior regeneration in *Aricia foetida*, figures and records a series of events apparently identical with those occurring in *Chaetopterus*. The active cells Probst calls 'Regenerationszellen.'

II

It has been maintained by several authors that germ cells in annelids have a common origin with one or other type of tissue cell—that they are not, in fact, early segregated from the somatic cells.

Cuénot ('91) states that in polychaetes in general one single gland produces both lymphocytes and germ cells, and that the two are therefore homologous.

Racovitza ('94) describes in *Micronereis variegata* relationships precisely as stated in the present instance. He describes the fact that the young somites are provided as they are formed with a cellular mass, from which amoebocytes and germ cells are developed.

Ruderman ('11) states that the genital cells of *Ephesia gracilis* are formed at the expense of amoebocytes.

Romieu ('23) remarks that it is difficult in many polychaetes to distinguish, in the early stages, the lymphatic glands and the gonads.

Nusbaum ('05, '08) and Iwanov ('08) maintain that the germ cells are distinct from the other embryonic cells.

Prenant ('29) also condemns the various authors who find identity between germ cells and amoebocytes, etc., or who have derived one from the other.

Malaquin ('25) established the identity between germ cells and neoblasts in *Salmacina dysteri*.

There is, therefore, in previous literature considerable agreement and some confirmation of the conclusions that in the case of *Chaetopterus* the germ cells are not segregated from somatic cells before the other tissues of the somite are differentiated, and that they have a common origin with the other tissue cells. These conclusions have a close bearing on the question of regeneration of gonads, as they indicate that there is no histological reason why gonads should not be regenerated as easily as any other organs.

III

It remains to consider how regenerative growth is related to the other phases of growth activity as classified arbitrarily above. The items in the list will be considered in order. (See list, page 24.)

1. Iwanov ('28) gives the most recent account of the histology of embryonic development in polychaetes and a full review of previous work. He concludes that regenerative growth is precisely similar to growth of the postlarval embryo. His figures of young *Eupomatus* resemble, almost exactly, both sections through regenerating growing points of *Chaetopterus*, and also sections of the growing point of adult *Filograna* (Faulkner, '30). Conditions in spionids are said to be different, although the figure of a regenerating *Nerine* seems to be essentially similar.

Iwanov interprets his results as in his earlier papers, and maintains that the mesoderm cells arise in the ectoderm and wander from their seat of origin into the interior. His argument is briefly stated on page 70 so:

. . . . die neuen Mesodermzellen, die den mesodermalen Somiten der post-larvalen Segmente den Ursprung geben, in die Liebeshöhle aus dem Ektoderm der Wachstumszone—der Ort ihrer Entstehung—hineinwandern. Im Zusammenhang mit dem Ektoderm dieses Bezirks ist auf Präparaten so deutlich zu sehen, dass die Voraussetzung einer jeglichen anderen theoretisch möglichen Entstehungsweise derselben ausgeschlossen ist, ...

This conclusion seems hardly justified. The connection between mesoderm and ectoderm is obvious, but it does not necessarily follow that A arises from B rather than B from A. nor is it certain whether the wandering cells are migrating into—or out of—the epidermis.

3. In *Filograna implexa* the regenerative growth associated with fission is achieved entirely by the activity of neoblasts (Malaquin, '93-'25, and Faulkner, '30), and in this species the events are identical—mutatis mutandis—with those occurring in *Chaetopterus*.

4. Potts ('11) states that the collateral buds in *Trypanosyllis* originate from an extracoelomic cellular proliferation.

5. Stolte ('29) gives an account of the somatic changes associated with sexual maturity in *Polyophthalmus pictus*, and although he does not emphasize the histological features, he states that he has been unable to trace the origin of the wandering mesodermal cells which are active at this time, in connection with the degeneration of muscles and the reorganization of the cellular layers of the body wall. The single mitosis which he saw was on a dissepiment, and it is possible that the cells are homologous with the wandering cells which are active in degeneration and regeneration in such genera as *Filograna* and *Chaetopterus*.

2, 6, and 7 have been previously discussed.

Some of the earlier authors discussed the question whether regenerative growth was a repetition of ontogenetic growth; actually there can be no question of repetition in two events starting from such different origins. Regeneration is rather a continuation of the processes of normal growth—though perhaps an exaggeration of them.

Furthermore, there is the much discussed question of germ layers and regeneration. The difficulties arising here seem to have been created by the nomenclature and aided by the attempt to correlate developmental and regenerative growth.

Germ layers—or equivalent differentiation of epithelia—do not exist as such in a regenerating growing point. The tissues at the time of their proliferation and origin are all confluent and homologous—and the early regenerated lobe is a homogeneous cell mass. The non-differentiated cells composing it, topographically and functionally, stand in precisely the same relation to all three of the germ layers. They occur among epidermal, intestinal, or mesodermal tissues, and are able to produce any tissue irrespective of its theoretical origin.

It happens during regeneration that some of the tissues differentiated in this cell mass are visible before the others. The intestine is present from the first and is the least changed during regeneration; then the epidermis is laid down first among the new tissues (compare figs. 4, 5, and 6). However, to conclude from this that the later appearing mesoderm (i.e., muscles, etc.) arises from the ectoderm seems to be an interpretation that confuses the essential simplicity of the facts. It is impossible to say at what time a particular cell assumes the characters of epidermis, and the simplest statement of facts as observed in *Chaetopterus* is that all the cells in the regenerated papilla are originally equipotential and embryonic, and that they are later differentiated into one or other type of tissue cell, according to their final location in the papilla. Hence the statement that muscles or other mesodermal structures arise by inward migration of epidermal cells is considered not relevant. In the case of species in which the neoblasts play a less complete part in regeneration, then the relationships of germ layers may have a different importance.

The review of the literature indicates that processes of growth and regeneration in polychaetes are perhaps histologically more uniform than might be expected from the great

diversity of external appearances. They are phases of the annelidan type of growth, which is a special property of metamerically segmented animals, and which is fundamentally dependent upon the existence of neoblasts. Such a type of growth is responsible for the extreme flexibility of growth activity and for the wide variations possible in body structure. As a result of the repetition of organs, it is anatomically possible to remove individual units without serious effect, to lose units or to insert them, and to produce them at any time and in any place, and, moreover, to retain an organic and functional individuality throughout, without physiological disturbance. And as a result of the ubiquity of multipotent cells, it is possible to reorganize tissues and organs after their original formation, to rejuvenate them on the same pattern, or to replace them in an altered form.

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CYTOPLASMIC STRUCTURES IN THE GANGLION CELLS OF CERTAIN ORTHOPTERA, WITH SPECIAL REFERENCE TO THE GOLGI BODIES, MITOCHONDRIA, 'VACUOME,' INTRACELLULAR TRABECULAE (TROPHOSPONGIUM), AND NEUROFIBRILLAE

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TWO HELIOTYPE PLATES (NINE FIGURES)

AUTHORS' ABSTRACT

The Golgi bodies of the nerve cells of the grasshopper are discrete elements composed of an osmophilic cortex and an osmophobic medulla. When seen in face, they appear ring-like, semicircular, or crescentic. The osmophilic portion is interpreted as the homologue of the classical Golgi apparatus and may under certain conditions be dissolved away, leaving clear spaces homologous to the canalicular apparatus (negative image of Golgi apparatus). When the osmophilic portion is dissolved, the osmophobic portion remains as a definite structure which apparently has no homologue in the classical Golgi apparatus. The mitochondria are granular, rod like, or filamentous and show no transformation into Golgi bodies. Neutral red bodies ('vacuome') of two kinds have been observed, neither of which is a constant preformed structure or has any relationship to the Golgi bodies. Prolongations of the capsule (trophospongium?) of the nerve cell penetrate into the cell approximately one-third the distance to the nucleus. These are interpreted as supportive rather than nutritive in function. A basket-like net of neurofibrillae surrounds the nucleus. It is suggested that the networks described as Golgi apparatus in certain invertebrate nerve cells may really consist of these neurofibrillae.

The conclusion of Parat that Golgi material consists either of mitochondria or 'vacuome' is rejected. The idea that all structures in the living cell which stain with neutral red ('vacuome') are homologous is also rejected.

Long ago, evidence was presented, mainly through the efforts of Golgi, Kopsch, Sjövall, Cajal, Von Bergen, Pensa, Deineka, Perroncito, Weigl, and many others, that the Golgi material in varying form is present in many types of cells. Furthermore, it was shown, by the beautiful researches of Hirschler ('18) and of Gatenby ('19), "that the Golgi elements in the segmentation of the egg are equally distributed and pass through development without losing their identity." In like manner it has been demonstrated that in many cases at least the Golgi material divides during mitosis (dietyokinesis), and is distributed to the daughter cells in a somewhat orderly manner.

Investigators, chiefly in Nussbaum's laboratory in Prague, came to the conclusion that the Golgi material is present in many invertebrate cells in a variety of forms, the most common of which are discrete bodies (granules, curved rods, banana-shaped filaments), and simple or incomplete networks. More recent investigations on this form of the Golgi material have added the interesting fact that many of the Golgi bodies are made up of two components, namely, an osmiophilic and an osmiophobic portion. It seems pretty generally agreed among most of the students of the cytoplasm who hold to the classical conception of the Golgi material that the osmiophilic portion of the duplex Golgi body found in many invertebrate tissues is really the homologue of the classical Golgi material. However, they are not all of the same opinion as to the nature of its osmiophobic portion.

This interpretation of the Golgi material in invertebrate tissue, both somatic and germinal, has not been left unchallenged. Parat has brought forward the idea that there are only two living cytoplasmic components, the 'vacuome' and the chondriome. According to him and his followers, the 'vacuome' consists of all structures which stain vitally with neutral red; the chondriome, of all structures which stain vitally with Janus green. The Golgi material, according to his interpretation, is an artifact, in some cases represented by the fusion of the neutral-red bodies or anastomosing 'active' chondriosomes and in others by hypertrophied and obese chondriosomes. In general, the former view holds for Golgi material in the form of a net and the latter for Golgi material in the form of discrete bodies. The latter conception, as pointed out by Gatenby ('31),¹ is not new, since the earlier investigators quite generally confused what we now call Golgi bodies with other cytoplasmic constituents. The views of Parat have been vigorously supported by other workers in France, among whom might be mentioned Hosselet, Radu, Dornesco, Zweibaum and Elkner, and others.

¹ Gatenby ('31) has recently given an excellent critical summary of the development of Parat's numerous views.

Voinov ('28) seriously objects to the interpretation that the Golgi bodies are homologous to the Golgi-Cajal network. He insists that they are universal and discrete structures in cells bearing no genetic relationship whatsoever to the Golgi material. Because of their important physiological function (?) in the cell, Voinov proposes the term 'ergastoblasts,' which denotes function rather than retaining the term dictyosome or 'lepidiosome,' which according to him refers to their morphological character only.

Holmgren ('00) long ago found in the nerve cells of *Helix pomatia*, and in many other types of cells, that definite prolongations of the surrounding stellate interstitial cells penetrate into the cytoplasm and there ramify to form a network. The prolongations become hollow, and form networks of canaliculi (*Saftkanälchen*), which open externally into the substance of the interstitial tissue. Holmgren considers the nerve cells of 'high' physiological importance which, according to him, must possess a trophic system that is realized in the form of a nourishing net of '*Saftkanälchen*.' Holmgren ('14) has insisted that the trophospongium and *Saftkanälchen* are identical with the positive and negative images of the apparatus of Golgi.

Finally, we have the suggestion of Walker and Allen ('27), Just and McNorton ('27), and Tennent, Gardiner, and Smith ('31) that the microchemical methods used by them on a study of the nature of the Golgi bodies did not permit them to differentiate between the Golgi material and non-cellular lipoids. In other words, they are of the opinion that the Golgi bodies and mitochondria are only inert non-living inclusions of the cytoplasm—a view which differs quite remarkably from the classical conception.

At no time has the discussion been more acute than it is at present, and the time seems ripe for a reinvestigation of this subject in an endeavor to substantiate or invalidate certain current views. However, it must be conceded, for the present at least, that, notwithstanding attempts to prove the Golgi material as pathological formations, as artifacts, as

optical illusions, as 'vacuome,' as non-cellular 'stuff,' or as hypertrophied and disguised chondriosomes, it is rapidly acquiring an important position among the components of the cytoplasm and demands a more critical consideration than has heretofore been generally given it.

One of us (Beams, '31) has previously considered the identity and genetic relationship of the cytoplasmic components as they exist in the vertebrate spinal-ganglion cells, and it is our purpose in this study to extend to the ganglion cells of certain invertebrates a similar study in an attempt to arrive at some general understanding of this much confused branch of cytology.

MATERIAL AND METHODS

The material used in this study consists of nerve cells from the thoracic ganglia of the following species of Orthoptera: *Arphia sulphurea*, *Chortophaga viridifasciata*, *Hippiscus apiculatus*, *Melanoplus differentialis* (Acrididae); *Gryllotalpa hexadactyla*, *Gryllus assimilis* (Gryllidae); *Blatta orientalis* (Blattidae); *Ceuthophilus* (Tettigoniidae), and *Tenodera sinensis* (Mantidae). The observations recorded here refer specifically to *Melanoplus differentialis*, although parallel series of observations have been made on *Blatta orientalis*. No attempt has been made to collect a complete series of observations for the nerve cells of the other species listed, since it was early evident that conditions were generally comparable to those in *Melanoplus differentialis* and *Blatta orientalis*.

The animals used in this study were all apparently in good physiological condition, many of them having been killed immediately after being collected in the field. In most cases only the thoracic ganglia were fixed, while in other cases the entire nerve cord with its complete set of ganglia was prepared.

The methods used to demonstrate the Golgi material consist of the Mann-Kopsch (Weigl) as modified by Ludford ('26), Nasonov, Cajal, Da Fano, Flemming fixative followed

by hematoxylin staining, and the method of Dietrich, Parat, and Volkonsky. The best and most consistent results were obtained by the methods of Nassonov and of Ludford. For the demonstration of the negative image of the Golgi material, the picroformol fixative of Bouin followed by iron-hematoxylin staining gave good results. Only fair results were obtained by fixation in 10 per cent formalin before staining with iron hematoxylin. The general fixation in the latter case was not as good as that obtained by the picroformol mixture of Bouin. In some instances following the method of Regaud the complete Golgi body was stained, while in other cases only the osmiophobic substance was differentiated and the osmiophilic portion of the Golgi body was dissolved away, leaving behind only a clear space marking its former position.

To disclose the intracellular trabeculae (trophospongium of Holmgren?), the formol-potassium bichromate-hematoxylin method of Regaud ('10) gave excellent results. Also, the intracellular trabeculae could often be seen in sections prepared according to the chrom-osmic method of Nassonov.

The mitochondria were revealed by the vital method of Janus green B and the permanent techniques of Regaud and of Bensley. In an attempt to demonstrate the Nissl bodies (chromatic substance), the method of Delaney ('27) and that of Feulgen were employed. The 'vacuome' was demonstrated by injecting 1 per cent solution of neutral red dissolved in physiological salt solution into the body cavity, and after a number of hours the ganglia were dissected out and examined in sealed preparations. The ganglia were also dissected out and mounted in salt solution containing a low concentration of neutral red. The neurofibrillae could sometimes be clearly seen following the method of Regaud ('10).

The grasshopper material has proved excellent for a study of the Golgi bodies (Golgi complex), negative image of the osmiophilic portion of the Golgi bodies, intracellular trabeculae (trophospongium of Holmgren?), mitochondria, the 'vacuoles' of Smallwood and Rogers, and the more recent

'vacuome' of Parat. The material is easy to obtain and reacts to most of the methods for the demonstration of the cytoplasmic components in a favorable manner. Therefore, we believe it worth while to suggest that the grasshopper ganglion cells offer unusually favorable material with which to demonstrate the Golgi bodies in a beginning course in microscopic anatomy and technique.

DESCRIPTION

1. Structure of the ganglia and distribution of cytons

The ganglia in the thoracic region of the grasshopper are composed microscopically of two parts, i.e., a cortical portion which is composed chiefly of ganglion cells and a medullary portion ('Punksubstanz'), composed mainly of straight and interlacing fibers together with a considerable amount of granular substance. The cortical portion of the ganglion is surrounded by a clear protective layer subjacent to which rests a layer of cells containing pigment and a fibrous connective tissue in which the cytons are embedded.

The cells in the ganglia of the central nervous system of the grasshopper show a marked variation in size. For convenience of description they may be grouped into three fairly distinct classes as regards size, namely, small, medium, and large. The chief underlying difference between the cells of the three classes of ganglia that are of particular interest here is in the somewhat marked variation between the ratio in nuclear and cytoplasmic volumes. Other more detailed differences, particularly those pertaining to the cytoplasmic components, will be pointed out from time to time in the later development of the paper.

2. Golgi bodies

Before taking up the detailed description of the Golgi material as it exists in the ganglion cells of insects, a word may be said concerning nomenclature. We propose to use the term Golgi body to indicate the scattered condition of the Golgi material in preference to the term dictyosome, a word used

by Perroncito ('10) to denote the bodies resulting from the fragmentation of the Golgi apparatus preceding its division (dictyokinesis) during mitosis. The Golgi body with which we are here concerned represents something more than the dictyosome of Perroncito. That is, in the grasshopper ganglion cells the Golgi bodies not only contain an osmiophilic portion (dictyosome of Perroncito), but an osmiophobic portion as well; the two taken together constitute the 'Golgi complex' of Bowen. The Golgi bodies in insect spermatogenesis also fragment into dictyosomes during cell division (Bowen, '20).

The Golgi bodies in the ganglion cells of the grasshopper (figs. 1 and 2) present a typical arthropod Golgi element consisting of a flattened plate-like osmiophobic medulla, bordered by an osmiophilic rim which we believe is homologous to the classical Golgi material. When seen in face, the Golgi bodies may appear like rings, semicircles, or banana-shaped structures, always possessing in favorable preparations distinguishable osmiophilic and osmiophobic portions. In profile these bodies appear more or less as straight rods and filaments, although all variations between the typical in face and profile views are found.

The distribution of the Golgi bodies in the large and medium-sized ganglion cells in general seems more or less at random throughout the cytoplasm (figs. 1 and 2). In the very small cells, however, their position appears limited, for the most part at least, to an intermediate 'layer' of the cytoplasm between the nucleus and the cell periphery. However, no definite orientation as regards the osmiophobic or osmiophilic portions of the Golgi bodies to the nucleus is apparent. Likewise, rarely, if ever, are the Golgi bodies found in direct contact with either the membrane of the cell or that of the nucleus.

There are fewer Golgi bodies in the small ganglion cells than in the larger cells, but the ratio in volume of the Golgi material and cytoplasm appears to remain, within rather wide limits, similar. However, the variation in form of the Golgi bodies seems much greater in the cells of larger size. We are

not prepared to present data at this time on the question of the multiplication of the Golgi bodies.

No concentration of the Golgi material in an eccentric juxtannuclear position in the small ganglion cells of adult grasshoppers, as described by Brambell and Gatenby ('23) for the small (young) cells of adult *Helix*, could be found. In our material it seems doubtful that the small ganglion cells represent young cells; but on the other hand, it seems more likely to us that the great variation in size of the ganglion cells is probably one of a functional significance rather than of age. This interpretation would seem to be in harmony with the view that very few, if any, new nerve cells are formed after embryonic development is completed. Furthermore, in all our observations to date on the ganglion cells of adult grasshoppers we have not been able to observe a cell in the process of division. In like manner in none of the preparations examined, whatever the technique employed, were we successful in finding an archoplasmic sphere. Accordingly, the absence of a demonstrable archoplasmic sphere suggests to us a direct correlation with the absence of an eccentric centralized Golgi apparatus as found in the small cells of *Helix* (Brambell and Gatenby, '23). If, therefore, this assumption and observation agree with the facts, then the discrepancy in the appearance of the Golgi bodies in the small nerve cells of *Helix* and those of the grasshopper probably inheres in the presence or absence of an archoplasmic sphere.

In preparations fixed in Bouin's solution and stained in iron hematoxylin the osmiophilic portion of the Golgi body appears as a clear unstained space (fig. 4). The osmiophobic portion of the Golgi body shows faintly, but in favorable preparations it can be readily observed. The clear spaces (negative image of the osmiophilic portion of the Golgi body) appear in every detail comparable as regards form and distribution in the cell to the positive condition. Figure 3 is taken from a slide prepared according to the method of Regaud. The osmiophobic portion of the Golgi

body appears as a well-defined mass typical of 'idiosomic material.' At the periphery of the osmiophobic portion is noted the negative image of the osmiophilic portion of the Golgi body. A similar condition has been reported in the nerve cells of *Helix* by Brambell and Gatenby. Such preparations, it seems to us, clearly demonstrate the unmistakable presence of the osmiophobic portion of the Golgi body and offer strong support to the view that the negative image of the osmiophilic portion of the Golgi body is identical with the canalicular apparatus of Bensley and of Cowdry, which is generally interpreted as the negative image of the apparatus of Golgi. This is to say, that the osmiophilic portion of the Golgi body has been dissolved away in the process of the preparation of the slides, leaving behind their cytoplasmic mold which appears as a clear space in the cell.

3. Mitochondria and neurofibrillae

The mitochondria in the ganglion cells of the insects do not differ greatly from those in the nerve cells of other animals (Ross, '22; Cowdry, '14; Brambell and Gatenby, '23; Sokólska, '30; Beams, '31, and others). They appear typically as granules, short rods, and filaments that are fairly evenly distributed throughout the cytoplasm of the cell (figs. 5 and 6).

No evolution of the mitochondria (hypertrophy) to form the Golgi bodies as figured and described by Parat ('28, '29), Hosselet ('29), Radu ('30), and others could be found. Furthermore, such complicated anastomoses of the mitochondria to form a reticulated network ('Golgi apparatus') so clearly figured by Hosselet in the ganglion cells of insects were never observed by us. We are at a loss to interpret the findings of Hosselet unless perhaps the network of structures figured by him as mitochondria are in reality fragments of the neurofibrillae. We have found in some of our preparations by the method of Regaud that the neurofibrillae are beautifully shown. They appear as a rather complicated basket of fine anastomosing filaments occupying a consider-

able portion of the cytoplasm (fig. 9). When seen in sections, the fine neurofibrillae might easily be mistaken for anastomosing filamentous mitochondria similar to those figured by Hosselet.

Ross ('22) has recently studied the neurofibrillae in the nerve cells of the crayfish and concludes "that it is highly improbable that the neuroplasm of the crayfish nerve cells contains either a fine or a course network such as revealed by a majority of neurofibrillar methods." From our observations on the neurofibrillae we are inclined to differ with Ross in so far as the neurofibrillae are considered to be artifacts. We can see no good reason for assuming that the neurofibrillae are artifacts and our evidence substantiates the view that the neurofibrillae are real neuroplasmic structures in the grasshopper ganglion cells. However, as to their function, whether conducting structures for nerve impulses or simply supporting structures, as suggested by Szüts ('14), we are not prepared to say.

As regards the suggestion brought forward by Parat that the mitochondria hypertrophy and give rise to the Golgi bodies we cannot agree. We have found no evidence that could possibly be interpreted to support Parat's conception, and while it may very well be assumed that the mitochondria and Golgi bodies are closely related, we are convinced from our studies that the proposed relationship figured and described by Parat, Hosselet, and Radu does not represent the actual condition of affairs.

In some of our preparations following Regaud's technique (fig. 5) we have found large oval bodies which vary in size and which we believe are comparable to the glycogen (?) bodies described by Kolatchev in *Limnea stagnalis*.

4. '*Vacuome*' of Parat

It has been known for a long time (Smallwood and Rogers, '07, '08) that vacuoles of various kinds can be demonstrated in the nerve cells of invertebrates. However, it has always been a debated question as to whether or not they represent

permanent preformed structures or are simply temporary formations in the cell.

Following the injection of neutral red into the body of the grasshopper, definite neutral-red bodies make their appearance in favorable preparations in the cytoplasm of the cell (fig. 8). These bodies vary in number and size, depending upon the strength, amount of the neutral-red solution injected, and time it is permitted to act. The neutral-red bodies are definitely of granular or globular type; their contours are often of irregular form, and their distribution is, in so far as we can determine, fairly even throughout the cell. These bodies represent, we think, aggregated dye particles. They conform to the description of the neutral-red bodies described by Ludford and by Beams for certain tissue cells of mammals and probably represent, as assumed by Ludford and by Beams, new formations in the cell.

When intravitaly stained neutral-red tissue (showing neutral-red granules) is subjected to treatment with Ludford's modified Mann-Kopsch technique, the neutral-red bodies are impregnated by the osmic acid and colored a deep black or brown. There seems little doubt to us that the impregnated granules are simply the neutral-red bodies fixed and impregnated by the Ludford technique. That these neutral-red osmiophilic bodies might possibly represent disturbed Golgi material, as suggested by Ludford and Gatenby for vertebrate tissue, seems rather improbable to us, since the Golgi bodies appear in every way characteristic in tissue from animals that have been heavily injected with neutral red.²

Besides the above-described type of neutral-red body in the grasshopper ganglion cells, there may be induced a second type of neutral-red body (neutral-red vacuole). We have noted with remarkable clearness the formation of a pink-staining vacuole surrounding the neutral-red granules in cover-glass preparations in which the tissue has been per-

² One of us has reported that in the nerve cells of rats injected with neutral red the Golgi apparatus is frequently broken up, although this is by no means invariably the case.

mitted to stand for twenty to thirty minutes. The longer the tissue is allowed to stand, the more numerous and larger the vacuoles become. The vacuoles appear similar to those described by Covell and Scott ('28) and noted by Beams ('31) in spinal-ganglion cells of mammals. The fact that vacuoles are seldom apparent when the preparations are first made and that they gradually increase in size the longer they are permitted to stand suggests to us that they are perhaps the result of katabolic action of the cell. This is substantiated by the fact that they are not impregnated in our preparations by osmic-acid methods which are fixed immediately after they are taken from the body of the animal. Furthermore, when spinal-ganglion cells are examined supravitaly with neutral red not until twenty to thirty minutes after the stain has been applied in favorable preparations can one usually see the appearance of vacuoles which tinge with neutral red. These vacuoles are, we think, destructive products of the cell, and their time of appearance can be regulated to some degree by the state of the environment. We are supported in this assumption by the following facts: 1) Vacuoles can be induced in the ganglion cells without the application of neutral red. 2) Vacuoles that have been induced will stain immediately with neutral red. 3) The vacuoles increase in number and in size the longer they are permitted to stand within certain limits. 4) This is comparable to what is known to be true for induced bodies in the erythrocyte of *Necturus* blood (Dawson, '29; Beams, '30 c). 5) They can be impregnated by Ludford's modified Mann-Kopsch method only after they have been induced. 6) They rarely, if ever, appear in properly fixed and impregnated material not previously treated with neutral red.

Therefore, it would seem from the foregoing facts that the neutral-red bodies in our material are quite separate from the Golgi apparatus, notwithstanding the dogma of Parat and his followers. This conception is, for the most part in agreement with the studies of Nassonov ('26), Chlopin ('27), Alexenko ('30), Ludford ('30, '31), Beams ('30, '31), and

others, i.e., that the neutral-red bodies observed simply represent new formations in the cell, either by the segregation of the injected neutral red into droplets or by the accumulation of waste products in the cell which are readily stained by neutral red. Just how this phenomenon is accomplished is still a debated question.

We have not observed in our material that the neutral-red bodies are formed in the substance of the osmiophobic or the osmiophilic portion of the Golgi bodies. As a matter of fact, the Golgi bodies show no signs of a fragmentation in the ganglion cells after the animal has been heavily injected with neutral red. This would seem to indicate that the injection of the neutral red into the living animal in this particular case has very little effect on the Golgi material either as a possible toxic agent or as a secretory stimulant. However, for the present at least, we do not wish to be interpreted as categorically denying the possibility of the Golgi bodies secreting the neutral-red bodies, but only to point out that our observations do not uphold this interpretation.

5. *Paracellular capsule and intracellular trabeculae* (*'trophospongium'* of Holmgren?)

Surrounding the cytone in the central nervous system of the grasshopper is a very prominent connective-tissue capsule. This capsule gives rise to many trabeculae (*'trophospongium'*) which penetrate into the cytoplasm of the cell (figs. 6 and 7). The trabeculae are very prominent in the cells of large size and can be easily distinguished in favorable preparations in the very smallest cells. At the point of origin of the intracellular trabeculae from the capsular fibers the filaments appear very robust and heavy. As they penetrate into the cytoplasm of the cell the fibers give off branches, many of which appear as very fine filaments. These dendrite-like structures generally penetrate a distance approximately one-third the way from the periphery to the nucleus, becoming less distinct as they progress inward until their identity is completely lost to view.

A question of considerable importance has recently been raised by Ross ('15) in his suggestion that the intracellular trabeculae in the nerve cells of the crayfish are in reality incomplete partitions which permit a continuity of the cytoplasm. In our material the intracellular fiber-like structures seem to represent something more than mere delicate threads; but they are obviously not of the nature of marked partitions. They appear to us more of the character of flattened plate-like filaments which branch freely, particularly at the periphery of the cell. The capsular buds are seemingly of the same fibrous character as that of the paracellular substance.

This brings us to the question of the possible relation between the trophospongium of Holmgren and the intracellular trabeculae. Ross ('15) has unhesitatingly interpreted the intracellular trabeculae of the ganglion cells of the crayfish as the same structure as described by Holmgren for the nerve cells of *Helix* and speaks of the paracellular capsule and intracellular trabeculae as the 'trophospongium of Holmgren.' We are entirely in agreement with Ross that the paracellular capsule in the grasshopper ganglion cells represents in many details, at least, the figures of Holmgren ('00) proposed to show trophospongium.³ If, therefore, this assumed homology between paracellular capsule with trabeculae and trophospongium is correct, our interpretation as to the function of the trophospongium contrasts sharply with that of Holmgren.

Our preparations show clearly that the intracellular system of trabeculae is continuous with the capsular bundle which possesses at intervals 'mantle' or 'sheath' cells which are thought to be the 'trophocytes' of Holmgren. We have found no evidence to uphold the interpretation of Holmgren that

³It is apparent that the structures described by Holmgren as trophospongium and Saftkanälchen in a wide variety of cells are not all homologous structures. As previously pointed out for the spinal-ganglion cells of the rat, the trophospongium of Holmgren is apparently nothing more or less than the Golgi apparatus. Furthermore, it is apparent that the intracellular trabeculae bear no obvious relationship to the intracellular trachea described by Ross and Tassell ('31).

the trophosphonium becomes canalized and forms 'Saftkanälchen' that are connected directly with the lymphatic spaces and are in reality nutritive canals.

In some of our preparations clear spaces appear which have been observed by Brambell and Gatenby ('23), and by Smallwood and Rogers ('08) for the nerve cells of *Helix*. These spaces resemble those described by Penfield ('21) for the spinal-ganglion cells of the rat. They are probably of little significance and very likely do not represent lymphatic spaces as suggested by Smallwood and Rogers.

6. *Nissl substance*

The question of the presence or absence of the Nissl bodies in the grasshopper ganglion cells has been considered by us, and our evidence seems to indicate that the latter is the case. That is to say, we were unable by the methods employed (which demonstrate beautifully the Nissl substance in vertebrate spinal-ganglion cells; Beams, '31) to demonstrate a substance which we could interpret as Nissl substance of the vertebrate type. These results are in agreement with those of Floyd ('03), who was unable to demonstrate a structure which he could consider as homologous to Nissl substance of vertebrate tissue in the ganglion cells of the cockroach. However, Nissl substance has been reported in the nerve cells of *Periplaneta* by Bialkowska and Kulikowska ('11), in the nerve cells of crayfish by Ross ('22), and in the ganglion cells of *Helix* by Brambell and Gatenby ('23).

Ganglion cells treated according to the method of Feulgen as given by Ludford ('28) revealed no structures which might possibly be interpreted as chromatin material in the cytoplasm. Our observations seem to indicate, therefore, that if Nissl substance really exists in the ganglion cells of the grasshopper, it does not take the characteristic form found in the nerve cells of mammals.

DISCUSSION

It is a well-known fact that the discovery of the Golgi apparatus ('internal reticular apparatus') was due to the efforts of Camillo Golgi ('98), the celebrated Italian neurologist, who clearly figured the Golgi apparatus in the nerve cells of mammals. It was the general opinion of Golgi, his students, and others who worked on the Golgi material immediately after its discovery that the Golgi apparatus is characterized in the cell by its reticular form. Accordingly, it was upon this criterion that they chiefly based their evidence for its identification in the cell. However, this conception of the Golgi material as a reticular structure, while mainly true for many vertebrate tissue cells, was soon brought into serious question by some who focused their attention on the study of the Golgi apparatus of the cells of invertebrates. Here was found, in many cases at least, instead of the classical apparatus of Golgi, the Golgi material in the form of isolated bodies (granules, curved rods, and filaments). These observations led to the conclusion that the term Golgi apparatus, while well adapted for the classical form of the Golgi material, is a misnomer when applied to the Golgi material in the cells of many invertebrates. These findings have led to the present conception of the Golgi material which has recently been voiced by Bowen and, we think, is shared by most cytologists, i.e., that the Golgi material must be thought of as a substance, the exact modeling of which is subject to wide variation in the cell.

When one applies the methods that are so successfully used for the demonstration of the classical apparatus of Golgi to the ganglion cells of the grasshopper, he is immediately struck by the marked variation in form of the supposedly homologous Golgi materials. The Golgi bodies appear as above described in the ganglion cells of the grasshopper in the form of discrete bodies, and show no apparent evidence of ever forming a network (contrary to the finding of Hosselet for certain insects). However, there can be little doubt, it seems to us, that the osmiophilic portion of the

Golgi bodies is comparable to the classical Golgi apparatus. This notion is, of course, not new and is the general one shared by most cytologists (however, see Monti, '15; Ross, '22; Hosselet, '29, and Migliavacca, '30), and is based on the fact that the osmiophilic portion of the Golgi bodies reacts without exception, in so far as we are aware, to the methods used in the demonstration of the classical apparatus of Golgi and the canalicular apparatus of Bensley and Cowdry (negative image of the apparatus of Golgi).

Monti ('15), working on the nerve cells of arthropods, has questioned the observations of the Polish investigators, Bialkowska and Kulikowska ('11, '12), that the Golgi material is present in the nerve cells of arthropods as discrete bodies. In fact, she finds by careful study a very delicate structure comparable with Golgi's internal reticular apparatus which according to her must have been overlooked by the Polish investigators. Furthermore, she holds that there takes place a direct transformation of the mitochondria granules into the network of the Golgi apparatus. More recently, Ross ('22), working on the nerve cells of the crayfish, was unable to demonstrate a typical apparatus of Golgi. This is not surprising, since such a typical Golgi apparatus does not exist in the nerve cells of the crayfish. Nevertheless, Ross is willing to accept the findings of Monti, whose figures are, according to him, "the only ones which give Golgi apparatus pictures of arthropod cells which resemble those of other forms, and we may assume that the preparations of others are incomplete or unreliable as far as their cytoplasmic structures are concerned."

Very recently, Migliavacca ('30) has reworked the nerve cells of *Astacus* as regards Golgi apparatus and finds a typical apparatus of Golgi with anastomosing filaments that he thinks resemble the reticular apparatus described for the nerve cells of the fetal mammal. He disagrees with Monti's view that the Golgi apparatus and mitochondria are one and the same structure and he interprets the large evenly distributed apparatus found by Monti in *Astacus* as quite dif-

ferent from the internal reticular apparatus of Golgi. The large internal reticular apparatus of Monti, according to Migliavacca, probably represents only anastomosing mitochondria. The real Golgi apparatus is, he thinks, present in the form of a small compact network which is comparable in many respects to the small apparatus figured by Monti. Migliavacca considers the annular bodies (Golgi bodies) as a particular developmental stage of the chondriosomes, as a particular aspect of the reticular apparatus, or as a different structure entirely. However, he thinks the first of these suggestions as the most probable—a view also held by Parat and his students when the Golgi material is present as discrete bodies.

Finally, we have the contributions of Hosselet, who by his work on certain insect nerve cells strongly supports the Parat 1929 dogma, namely, that the mitochondria hypertrophy, giving rise to a network of chondriosomes which has, according to him, been mistaken by others for Golgi apparatus. A somewhat similar conception is held by Radu, who finds a gradual evolution of the mitochondria into dictyosomes in the tissue cells of *Armadillidium*. He does not record, however, that they fuse to form a typical apparatus of Golgi. Voinov and Athanasiu ('28), working on the nerve cells of the snail *Helix*, have in like manner added to this chaotic state of affairs by the important conclusion that for twenty years two cellular elements have been confused, namely, the ergastoblast (Golgi bodies) and Golgi-Cajal network.

It is evident from the foregoing description that our observations and interpretation of the Golgi material in the ganglion cells of the grasshopper sharply diverge from those of Parat, Monti, Voinov and Athanasiu, Migliavacca, and Hosselet. It is further evident that these investigators are not agreed among themselves as to what actually represents the Golgi apparatus. In our studies on the ganglion cells of the grasshopper we find the mitochondria to be composed of short rods and granules and never have we observed that they give rise to long anastomosing filaments. Furthermore,

the Golgi bodies in our material are always in the form of discrete bodies comparable in most respects to those described by Bialkowska and Kulikowska for the ganglion cells of insects and by Brambell and Gatenby for the nerve cells of *Helix*, all of which are typical of the Golgi bodies in arthropod cells.

Since our observations and interpretation of the Golgi bodies in the arthropod ganglion cells (e.g., grasshopper, crayfish) confirm the findings of Bialkowska and Kulikowska ('12), Brambell and Gatenby ('23), and of Sokólska ('30), and since they diverge so sharply from those of Monti ('15), Parat ('28, '29), Hosselet ('29), and Migliavacca ('30), we feel obliged to offer a possible explanation for the wide discrepancies of our views. However, since such an attempted explanation defies precise analysis, we can only venture the possible explanation that if the net-like apparatus described by Monti, Migliavacca, and Hosselet represents cytoplasmic structures at all, it is probably a part of the intracellular system of neurofibrillae. This is, we think, undoubtedly the case in the instance of Hosselet, and some of the preparations of Monti seem susceptible to an interpretation along the same lines. However the case may be, we question the evidence so far brought forward and the interpretations appended, namely, that the Golgi bodies are in any direct way related to the mitochondria in the manner urged by Monti, Hosselet, Parat, and others. But, quite to the contrary, we find the typical Golgi body that has been found so widely distributed in arthropod tissue. We are in agreement with Gatenby in that we too have never been disturbed in making a decision as to the identity of Golgi material and mitochondria. But, as Professor Gatenby ('31) has recently pointed out—

If and when any evidence of a convincing nature of the origin of the Golgi bodies from mitochondria is produced, all that will be necessary will be the recognition of the fact. In such cells as those of the pancreas acinus we are not sure as to the function of the mitochondria. But to call them inactive chondriome seems a little premature. In the same way, until we know that the Golgi apparatus is derived from mitochondria, to call the former active chondriosome seems equally unjustifiable.

A question of considerable cytological importance presents itself in the arthropod Golgi bodies as to the nature of the osmiophobic portion. The osmiophobic portion of the duplex arthropod Golgi element has been given various names by previous authors, some of which presumably indicate a homology of known components of the cell. For instance, Hirschler called it 'apparatinhalt,' Gatenby used the term archoplasm; Bowen and Duesburg, 'idiosome substance'; Papanicolaou and Stockard, 'idioendosome'; Sembrat, 'the internum,' and Parat, 'vacuome.' On the other hand, others such as Weiner ('30), Nassonowa ('27), and Morrelle ('27) have questioned the existence of such a substance and propose to explain the described appearance of the osmiophobic portion of the Golgi bodies as the result of optical illusion. Bowen made a wide search in vertebrate somatic cells in an attempt to find an osmiophobic portion of the Golgi material, but was unsuccessful. However, it is interesting to note that Sokôlska ('30) figures, in *Ciona intestinalis*, Golgi bodies which appear to possess an osmiophobic portion. The osmiophobic substance of the arthropod Golgi body seems always to be in intimate association with the osmiophilic portion (classical Golgi material), which suggests to us that it should be thought of in insect somatic cells, at least, as a part of the Golgi element rather than as the idiosome or archoplasmic substance which might be associated with the Golgi material.

If we define the term archoplasm and idiosome in such a way as Professor Wilson has done in the glossary of his book "The Cell in Development and Heredity," we find that the term archoplasm is "the substance of which consist, or from which arise, the spindle-fibers and astral rays; believed by Boveri to constitute a specific material; nearly equivalent to Kinoplasm." On the other hand, the term idiosome represents "a spheroidal cytoplasmic body in the early auxocytes, particularly in the spermatocytes, variously called by earlier writers the 'attraction sphere,' 'archoplasm-sphere,' 'sphere,' etc. It surrounds the centrioles and is itself surrounded by the Golgi apparatus (of which it may form a part) and often

also by chondriosomes." Now the question immediately arises: Is the Golgi complex in arthropod somatic cells the same as that in germ cells? MacBride and Hewer ('31) make the statement that the idioplasm (by which term they probably mean idiosomal material) "has only been observed in germinal tissue, and has not been demonstrated in somatic cells." This statement may be true in so far as mere definition is concerned, but we are convinced that the osmiophobic portion of the Golgi bodies is identical in both germinal and somatic tissue of insects. Gatenby ('19) clearly recognizes this fact when he states that ". . . there is no doubt that the archoplasm of both somatic and germ cells is identical." Therefore, it seems to us that the use of the terms archoplasm and idiosome is not the most desirable, since they do not accurately define the osmiophobic portion in somatic cells. Perhaps the terms osmiophile and osmiophobe or Golgi externum and Golgi internum, as suggested by Sembrat ('30), are the most desirable because of their more general application.

A more widely discussed and perhaps less plausible hypothesis by which to explain the nature of the apparatus of Golgi has been recently resurrected, mainly through the efforts of Parat and Painlevé, of Paris. In 1924, these investigators claimed that there had been discovered in animal cells a new cellular component in the form of a collection of neutral-red-staining granules and vacuoles. They assert that the precipitation of metallic compounds within, on the periphery of, and between the neutral-red bodies represents the Golgi apparatus of previous authors. Wherever a network is encountered it is due to the fusion of the neutral-red granules simulating the artifact known as the 'Golgi apparatus.' The general view of Parat and his associate received considerable favor among many cellular anatomists, and in spite of the enormous literature to the contrary, is still consistently urged by some (for instance, Dornesco, '31).

Considerable discussion has arisen in some quarters regarding the first suggestion made in animal tissue of the

'vacuome-Golgi apparatus' hypothesis as outlined by Parat and Painlevé ('24). It is not our purpose in this place to seek out the original source of this idea, but only to point out that it was clearly set forth in all its present details (Parat, '24, '25, '28) as early as 1907 and 1908 by Smallwood and Rogers, as is indicated from the following quotation:

Probably the commonest structure present in the cytoplasm of the invertebrate nerve cells is the vacuole. These vacuoles are present in all of the great groups already cited, although usually described under the terms 'lymph space,' 'Netzapparate,' 'Saftkanälchen,' 'Trophospongium,' etc. The vacuole can be determined in the following manner in the living cell: Isolate a nerve cell and study it in a 1-500 solution of methylene blue or neutral red in normal salt solution under the oil immersion lens. At first, but little can be determined: but as the stain progresses the vacuoles become more distinct and their contents often take on a differential stain. The experienced worker can make out these vacuoles without any stain. The time that it takes to stain these vacuoles will vary; but usually 5 to 20 minutes will be the limit, as after that time the nerve cell is apt to become overstained and undergo some changes in its general appearance and the character of its parts. This gives about 15 minutes when a critical study may be made. During this time the vacuoles are readily made out as isolated spherical bodies containing a fluid. It is impossible to trace any connection between vacuole and vacuole. The size is also further evidence of their individuality, for they range from the most minute bodies recognizable with oil immersion lens to structures a third the size of the nucleus.

Smallwood and Rogers further state that vacuoles occur in the nucleus and nucleolus that are probably formed in each case in a similar manner even if the exciting cause is different. They also state that the vacuoles are transitory structures, vary in number, and are not limited by distinct walls.

From our own studies on the 'vacuome' in the grasshopper nerve cells we are convinced that there are two types of neutral-red bodies which may be demonstrated in the living nerve cell, both of which we think represent new formations in the cell. First, following the injection of an aqueous solution of neutral red into the body cavity of the grasshopper,

small red granules of considerable variation in size and number make their appearance in the cell. These bodies are, we think, evoked by the neutral red injected. In other words, they are dye droplets comparable in every respect to the 'vacuome' of Parat as described by Beams ('30, '31), Ludford ('30, '31), Alexenko ('30), and others for the tissue cells of vertebrates and which have been interpreted by them as new formations in the cells. Secondly, if an excised ganglion is removed from the body of the grasshopper and is exposed to a relative dilute aqueous solution of neutral red on the slide, there will be noted after twenty to thirty minutes the appearance of small red-staining vacuoles in the ganglion cells comparable in every respect to those described by Smallwood and Rogers. This type of induction of neutral-red-staining vacuoles can easily be demonstrated in the erythrocyte of the frog as described by Jordan ('25) and Beams ('30). Such vacuoles appear in cells not exposed to neutral red and also around the neutral-red granules caused to appear by the injection of the neutral red. This phenomenon of induction probably results, as suggested by Cunningham ('30), from the accumulation of waste products, the abnormal environment being the stimulus. We are, therefore, unconvinced of the presence of a new cellular component in the nerve cell comparable to the vacuome of Parat. But more probably the vacuoles either represent new formation in the cell, as held by Chlopin ('27), Krjukowa ('29), Beams ('30, '31), Ludford ('30, '31), and Alexenko ('30), or are only temporary structures associated with the metabolic products of the cell. As pointed out by Gatenby, they cannot be considered in the same category as Golgi material and mitochondria.⁴

Professor Gatenby, while not convinced that the 'vacuome' of Parat represents new formations in the acinar cells of the pancreas as described by Covell, Beams, and Ludford, nevertheless states that "the appearance of two recent papers by

⁴In addition to giving rise to new formations ('vacuome') in somatic cells, neutral red will, of course, stain certain preformed structures in the cell.

Ludford and by Beams and Goldsmith, has done much to unsettle the previous views held by the present writer, and to make it seem very doubtful as to whether there really is anything in the nature of a separate constantly occurring 'vacuome' or vacuolar 'system'." Furthermore, while Gatenby's paper was in press papers appeared by Ludford ('31) and by Beams ('31) which further substantiate their previous conceptions, namely, that the Parat 'vacuome' in the tissue described by them simply represents segregated dye particles.

The question thus follows: Does the neutral-red substance enter the cell by way of the secretory route and is the Golgi material by any chance directly responsible for the aggregation of the ultramicroscopic dye particles into large visible droplets? In this connection considerable evidence has accumulated of late which goes to show that the neutral-red bodies are secreted by the Golgi bodies (Krjukowa, Ludford, Alexenko, Gatenby, and others). However, from our material we are not convinced that such is the case. In other words, we do not believe that the Golgi apparatus is always the synthetic center of the cell in secretion. This is substantiated, it seems to us, by the fact, as shown by Gatenby ('29), Monne ('30), Sembrat ('30), Hirschler ('28), and others, that the 'vacuome' may be located within, intimately associated with, or remote from, the osmiophobic portion of the Golgi bodies. Furthermore, we have not observed that the osmiophilic substance of the Golgi apparatus bears any marked relation to the neutral-red bodies. In our material the Golgi bodies were not broken up following the injection of neutral red, as was noted by Ludford and by Gatenby for tissue cells of vertebrates. While it seems probable, therefore, that the neutral red may enter the cell by way of the secretory route, it seems equally probable that it is not in all cases directly associated with the Golgi apparatus. Accordingly, we are of the opinion that the Golgi bodies are not the sole synthetic center of the cell and that they function as a part of the cell system, the cell as a whole participating in secretory phenomena.

Walker and Allen ('27), Just and McNorton ('27), Tennant, Gardiner, and Smith ('31) have recently experimented with a number of non-cellular substances (mayonnaise, egg white, etc.) by smearing them on slides and treating according to the current cytological techniques for the demonstration of the Golgi apparatus. They report that figures exactly like those of mitochondria and Golgi bodies can be obtained and they accordingly arrive at the important conclusion that mitochondria and Golgi bodies are only inert, non-living inclusions of the cytoplasm. We are not surprised at the findings of these investigators; but with their conclusions we cannot agree. Their observations, interesting as they may seem, when carefully analyzed really mean nothing beyond the fact that attempts have been made to manufacture mitochondria and Golgi material. We consider such efforts to explain the nature of the Golgi material and mitochondria in the same category as the efforts of Klein, van Beneden, Heidenhain, and others, to demonstrate the mechanics of mitosis by models.

We wish to express our appreciation to Mrs. Esther H. Powers for her painstaking care in making the figures.

CONCLUSIONS

1. The Golgi bodies (Golgi complex of Bowen) in the ganglion cells of the grasshopper consist of isolated elements which are composed of two components, namely, an osmiophilic and an osmiophobic portion. These Golgi bodies are typical of the arthropod elements which when seen in face give one the impression of ring-like, semicircular, or banana-shaped structures.

2. The osmiophilic portion of the Golgi bodies can be dissolved away, leaving behind the mold of which the Golgi material formed the cast. This condition parallels the demonstration of the canalicular apparatus (which is simply the negative image of the Golgi material) by Bensley and by Cowdry.

3. The mitochondria in the ganglion cells of the grasshopper are composed of granules, short rods, and filaments. They were never observed by us to hypertrophy and give rise to the Golgi bodies as claimed by Parat, Radu, and others, nor were they ever observed to anastomose, thus giving rise to a network similar to the 'Golgi net' as figured by Hosselet.

4. The neurofibrillae are composed of anastomosing threads of fibers which surround the nucleus. It is thought that some of the structures figured by Hosselet as modified mitochondria are probably part of the system of neurofibrillae.

5. Neutral-red bodies ('vacuome' of Parat) may be caused to appear in the ganglion cells of the grasshopper following the injection of an aqueous solution of neutral red into the living animal. The neutral-red bodies vary in size and number in the cell, depending to some extent upon the amount and concentration of the neutral-red solution injected and the time it is permitted to act. These bodies are considered new formations in the cell evoked by the neutral red injected. A second type of neutral-red body may be observed to appear in ganglion cells that are stained supravitaly. These vacuoles, which appear in excised tissue after twenty to thirty minutes' exposure to neutral-red solution, are considered the end product of katabolic action of the cell. The conclusion is reached that there are no preformed red-staining granules or vacuoles in the nerve cell of the grasshopper of the character described by Parat; neutral-red bodies present are either induced or temporary and are probably associated with the katabolic action of the cell.

6. The intracellular trabeculae (trophospongium of Holmgren) are described as emerging from the fibrous capsule of the cell and penetrating the cytoplasm approximately one-third the distance to the nucleus. The intracellular trabeculae are interpreted as fibrous structures which form a supporting framework for the nerve cells. Holmgren's interpretation that the intracellular trabeculae become canals for the passage of nutritive materials into the cell is thought to be in error. Also, Holmgren's notion that the Golgi material is

the same as the intracellular trabeculae (trophospongium) is rejected for insect nerve cells.

7. No Nissl bodies of the same character as those found in vertebrate nerve cells have been found by us in the nerve cells of the grasshopper.

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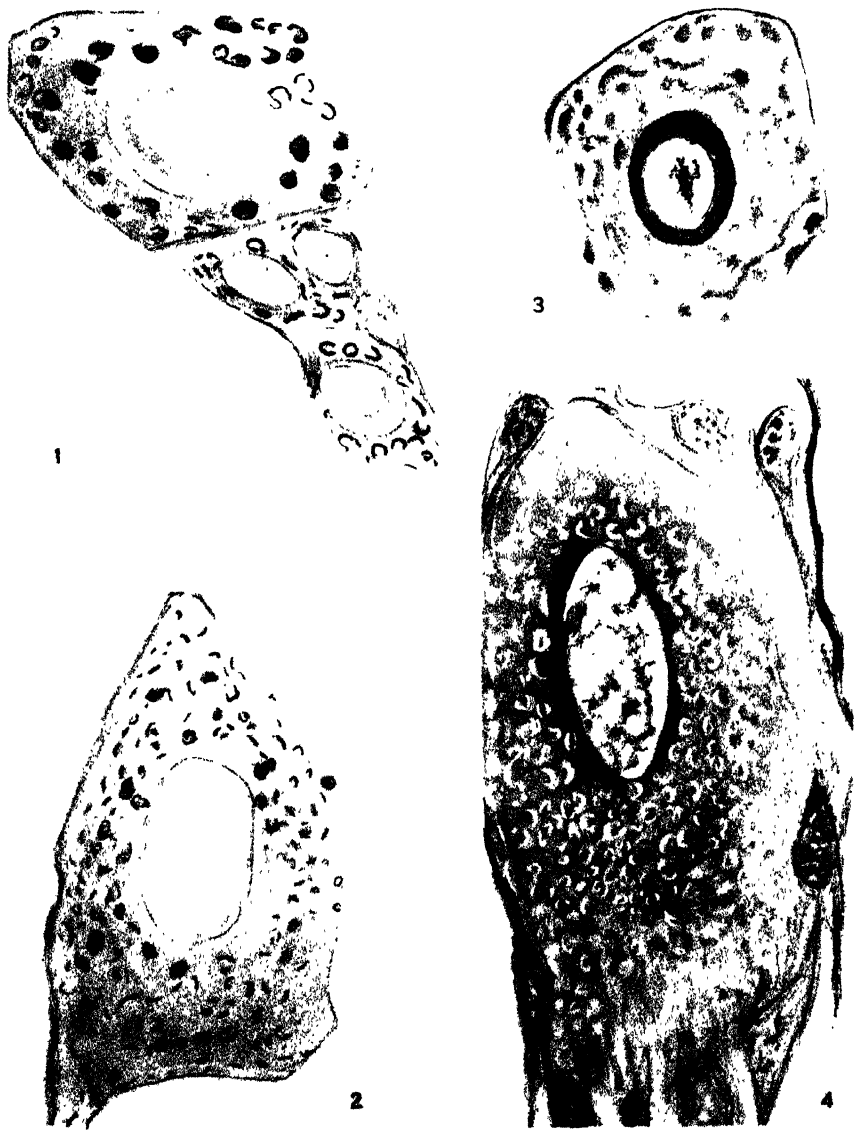


PLATE 1

EXPLANATION OF FIGURES

- 1 Small and intermediate ganglion cells, showing typical insect Golgi bodies. Nasonov.
- 2 Large ganglion cells showing large number of Golgi bodies. Nasonov.
- 3 Medium sized cell with osmophobic portion of Golgi bodies enclosed in clear spaces which represent the negative image of Golgi material. Regaud, iron-hematoxylin.
- 4 Large cell showing negative image of Golgi material. Bouin, iron-hematoxylin.

PLATE 2

EXPLANATION OF FIGURES

5 Medium-sized cell with mitochondria and bodies interpreted as glycogen (?). Regaud, iron-hematoxylin.

6 Large cell showing mitochondria and paracellular capsule with short intracellular trabeculae. Regaud, iron-hematoxylin.

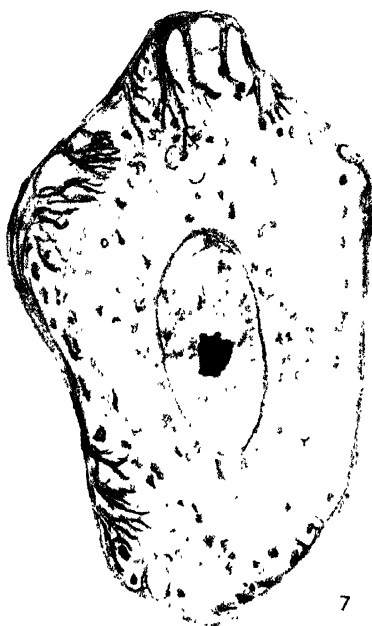
7 Large cell with Golgi bodies poorly preserved and paracellular capsule with relatively long intracellular trabeculae. Regaud, iron-hematoxylin.

8 Cells of medium size with neutral-red bodies ('vacuome') resulting after injection of neutral-red solutions into body cavity. Intravitaly stained.

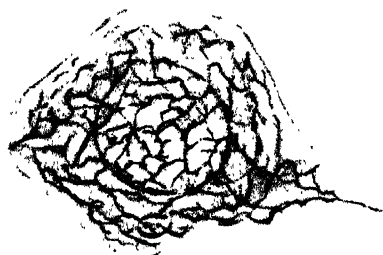
9 Cell of intermediate size with neurofibrillae. Regaud, iron-hematoxylin.



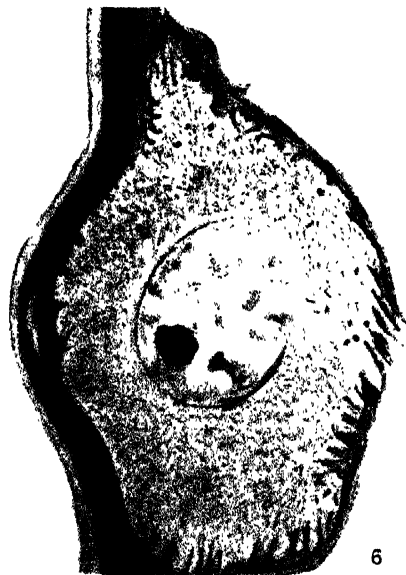
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SOME CYTOPLASMIC STRUCTURES IN THE MALE REPRODUCTIVE CELLS OF A PSEUDOSCORPION (CHELANOPS CORTICIS)

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SIX PLATES (SIXTY-FIVE FIGURES)

AUTHOR'S ABSTRACT

The chondriosomes of the germinal epithelium are vesicular, whereas the Golgi material takes three forms. The aggregation of chondriosomes about the Golgi cap, located on the nucleus, suggests a chemical interchange between these in the growth period. They become cylindrical as the Golgi cap moves from the nucleus and becomes spherical. It is at this time joined by other Golgi material. The entire aggregation is dispersed previous to the maturation divisions, where the inclusions studied are only approximately equally divided, in contrast to conditions in the true scorpion, *Centruroides exilicauda*. Golgi granules on chondriosomal surfaces in the spermatid again suggest chemical interchange. The later changes of chondriosome vesicles to discs, and back to spheres, may be means of regulating chemical reaction. A close relation between chondriosomes and Golgi material is indicated by various bodies, of Golgi origin, within the former in the spermatid. A thick thread finally originates from among the chondriosomes of the elongated sperm. Spiral threads surrounding the elongating nucleus arise from reunited Golgi material, a derivative of this substance not previously recorded. The acrosome, also of Golgi origin, undergoes several changes in shape and shows several parts. The elongated nucleus, spiral thread of Golgi origin, acrosome, chondriosomal thread, and unusually long axial filament become coiled and encysted in an oval mature sperm capsule. In the female duct the sperm uncoils.

The objectives in this study have been: First, to determine, if possible, something further of the function of the chondriosomes and Golgi material; secondly, to compare the

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behavior of the chondriosomes in the spermatogenesis of this form with that in the true scorpion; and thirdly, to observe what happens to the mature encysted sperm, characteristic of this group, after it has entered the reproductive duct of the female.

While this study was in progress, I. Sokolow ('26) published on the spermatogenesis of two European species of pseudoscorpions, *Obisium muscorum* and *Chelanops cyrneus*, in which he also studied the chondriosomes and Golgi material. Our work will be mutually beneficial in that it not only will give a check on observations, but will furnish a comparative study of species from opposite sides of the world.

MATERIAL

Approximately fifty species of pseudoscorpions representing eleven genera were recorded in the United States by Coolidge ('08). For this study, a total of 708 specimens representing fourteen species were collected. Of the 472 slides examined, 268 preparations were successful. Forty-nine *Chelanops corticis* specimens were used. These were collected at Bloomington, Indiana, and Urbana, Illinois. The remainder of the specimens studied were from localities as follows:

1. *Verrucaditha megaloptera* (Chamberlin). Bloomington and Winona Lake, Indiana.
2. *Chthonius tetrachelatus* (Preyssler). Bloomington, Indianapolis, Lincoln City, and Traders Point, Indiana.
3. *Chelanops dentatus* (Banks). Bloomington, Indiana.
4. *Chelanops oblongus* (Say). Bloomington, Culver, Indianapolis, Lincoln City, Madison, Mentone, Mitchell, Royalton, Smithville, Sullivan, Traders Point, Waveland, and Winona Lake, Indiana.
- 5, 6, 7. *Chelanops* (three new species, Chamberlin). (Related to *tumidus* and *oblongus*.) Culver, Indianapolis, and Royalton, Indiana.
8. *Chernes scorpioides* (Hermann). Bloomington, Indiana.
- 9, 10. *Chernes* (two new species, Chamberlin). Bloomington, Indiana.
11. *Chelifer caneroides* (Linnaeus). Bloomington and Winona Lake, Indiana.
12. *Chelifer* (one new species, Chamberlin). Winona Lake, Indiana.
13. *Chelifer muricatus* (Say). Bloomington, Clear Creek, Culver, Indianapolis, Lincoln City, Mentone, Mitchell, Royalton, Traders Point, Waveland, and Zionsville, Indiana; Urbana, Illinois.

The report was confined principally to the species *Chelanops corticis*, as the very capricious Golgi methods worked best on this material. The species from which cells are figured are listed with the description of the plates.

The collection of pseudoscorpions was very uncertain, due to the special type of habitat in which they were found.

Chelanops corticis lives in the ridges on the outside, and among the lichens which encrust the bark of hackberry and maple stumps. It is here well protected by the similarity of the color of its body to that of the bark. The species name well describes the superficial habitat.

Best fixations were obtained on material collected between July 2nd and October 17th. Female specimens of *Chelanops oblongus* were found with eggs as early as June 10th, so that sexual maturity probably occurs near that date. The latest date eggs were observed for any species was October 25th (*Chernes scorpioides*).

METHODS

Material was killed by first covering the animal with a very small drop of Ringer's solution, and then cutting off the abdomen. The contents of the abdomen were then carefully teased out. The chitin and contents were left attached, and the mass was fixed by immersion in the various fixing reagents, in 8-cc. glass-stoppered bottles in the dark.

The most successful preparations were made by the Kolatchev method as described in detail by Bowen ('28). I obtained best results by osmication in 2 per cent solution at 35°C. for eight days in the dark. It was not necessary to stain this material. The following methods of fixation also gave good results:

1. Fixation in Champy solution for twenty-four hours, and washing in twelve changes of distilled water during twenty-four hours.

2. Fixation for four hours in Benda solution, using three to four drops of acetic acid to 3 cc. of the mixture, and washing in four changes of distilled water during two hours.

3. Fixation by the Champy method as described by Gatenby ('20, p. 290), using pyroligneous acid and chromic-acid mordant, followed by bichromate solution.

4. The Mann-Kopsch method for the study of Golgi material.

5. Standard Flemming and Bouin fixation for the study of chromatin. (The chondriosomes were visible in Flemming fixations.)

All clearing was in xylol, embedding in 53° melting-point paraffin, and sectioning 4 μ in thickness.

With the exception of the Kolatchev and Mann-Kopsch methods, staining was in iron-alum hematoxylin, which was found more reliable than the acid-fuchsin or crystal-violet methods.

For a study of the coiling of the structures in the spermatid, to form the encysted sperm, smears were found useful.

For identification of chromatin, acrosome, tail, and other components in living cells, the Biondi stain was found very useful on material teased apart in Ringer's solution. The formula of Krause and his dilution ('26, p. 458, line 3, and fourth line from bottom of page, respectively) were used. It was found best to add enough stain almost to obscure the field, then to replace again with Ringer's solution. Since the stain dissolved again in Ringer's solution, it was necessary to keep a balance between Ringer's and Biondi while studying under oil immersion.

THE GOLGI MATERIAL AND ITS DERIVATIVES

The earliest stage to which I can trace the male reproductive cells is their formation from the germinal epithelium, which consists of a syncytium and surrounds the cysts of reproductive cells (fig. 1). This epithelium varies in thickness with the size of the enclosed cyst and is largest around early spermatogonia.

The Golgi material in the syncytium consists of irregular masses of small size, some net-like, others made up of a

lightly osmicated material with heavily osmicated granules embedded therein; while in the early spermatogonia it is made up of one or two small, irregular, heavily osmicated masses lying near the nuclear wall, and of other small, heavily osmicated granules dispersed in the cytoplasm (fig. 1).

At the stage in the early growth period when the chromatin begins to form threads preparatory to contraction (fig. 2), the masses of Golgi material have broken up into numerous small granules dispersed in the cytoplasm. Some of these particles later fuse to form, at one side of the nucleus, the large cap-shaped, lightly osmicated structure (fig. 3), while others remain dispersed through the cytoplasm or lie on the surface of the cap.

In the late growth period, that part of the Golgi material which was in the cap moves away from the nuclear surface, becoming spherical, with numerous black granules scattered inside, others located on its surface. The other part consists of two irregular, heavily osmicated masses lying outside the hull of chondriosomes which are grouped about the sphere (fig. 5). In Benda preparations the central sphere appears homogeneous, with one or two darkly staining dots inside (figs. 6, 7, and 8), which were interpreted as centrosomes by Sokolow ('26), but which I was unable to follow through as such. The attractive force which causes the chondriosomes to be grouped in a hull about the sphere would strongly suggest that we have here a center comparable to a centrosome or pole of a cell, in this case exerting its influence principally on the cytoplasmic inclusions.

In a still later stage of the growth period the two irregular masses of Golgi material outside the sphere have fused into one, while the sphere has become heavily osmicated with a layer of small granules surrounding it closely within the chondriosomal hull, other granules lying scattered between the chondriosomes or dispersed in the cytoplasm (fig. 10). In a stage similar to this, Sokolow ('26), working with the pseudoscorpion *Chelanops cyrneus*, believes there is a flowing of osmiophilic granules from the outer Golgi material to the

central sphere by means of a cord connecting the two. I found no such cord in *Chelanops corticis*, but occasionally a suggestion of the same in *Chelanops oblongus* (fig. 13). The small granules in *Chelanops corticis* (which I interpret as Golgi material) flow from the cytoplasm, between the chondriosomes on all sides, to the sphere until the cytoplasm is finally cleared of them (figs. 10 and 14).

After the chondriosomes have become dispersed, all the Golgi material, including the central sphere, fuses into one large mass consisting of a lightly osmicated matrix with lumps of heavily osmicated material embedded therein (fig. 14). This complete withdrawal and fusion of the Golgi material into one mass suggests that there is a slowing down of reaction between the material of which it is composed with other material in the cytoplasm since less surface would be exposed than in a diffuse condition.

Previous to the maturation divisions, the heavily osmicated material breaks up again and becomes dispersed throughout the cytoplasm as irregularly shaped particles and granules (figs. 15 and 16). In the divisions the Golgi material precedes the chondriosomes to the poles of the cell and tends to become grouped around the locations of the poles (figs. 19 and 20). A similar tendency is noted by Bowen ('20) in two species of Hemiptera, and Wilson ('25) in the scorpion *Opisthocanthus elatus*. Payne ('27) also observes an influence of the division centers on the Golgi material in the spermatocyte divisions in *Gelastocoris*. As is noted by Wilson ('25) in the scorpion *Opisthocanthus elatus* and by Sokolow ('26) in the two species of the pseudoscorpion, we have here again only an approximately equal division of this inclusion between the two resulting cells.

After the maturation divisions, the Golgi material reassembles near the nucleus into two and then into a single large mass composed of a lightly osmicated matrix, with heavily osmicated irregular masses and granules embedded therein (fig. 23). Some of the latter are also dispersed through the cytoplasm (fig. 21). There is a tendency for these particles

to adhere to the chondriosome surfaces (figs. 21, 23, and 24), and where they are few in number they seem to connect to the ends of lines in the chondriosomes (fig. 23). The granules in the cytoplasm are still present at the time the acrosome is started (fig. 28), and are grouped about it in some cells (fig. 32). Near this stage we first find, within the chondriosomes, spheres, and comma-shaped bodies which appear to originate from the granules on the surface of the chondriosomes (figs. 29 to 32), since the latter disappear with the formation of these new structures.

FORMATION OF ACROSOME

The main Golgi mass, which has become ovoid or cup-shaped with a thin, heavily osmicated wall enclosing a lighter substance, now gives rise to the acrosome (figs. 26, 27, and 28). Vesicular clear structures arise from the Golgi material earlier (fig. 24), but the acrosome has a direct origin from the latter. The portion of the cup which is adjacent to the acrosome and which is not so heavily osmicated as the remainder may represent an intermediate substance formed by the Golgi material, and in turn might give rise to the acrosome (figs. 26 and 27). An ovoid-shaped body, sometimes clear and in other cells with a gray substance inside, has a similar location in later stages (figs. 29 to 32 and 35). This body may take part in the formation of a cap about the chromatin (fig. 36) as mentioned later; however, a similarly shaped structure lies to one side of the acrosomal junction with the chromatin (fig. 42) after the cap is formed. Other small vesicles appear on the surface of the Golgi cup at almost any position while the acrosome is being formed (figs. 29 to 32 and 35).

Throughout the early spermatid, from the second maturation division to the time the acrosome begins to form, the chromatin has been in a diffuse condition. Just after the acrosome has begun to take on a cone shape, the chromatin begins to contract and condense so that it forms an irregular hemispherical body inside one end of the nuclear membrane (fig. 29).

The Golgi mass, which has been in contact with the nuclear wall at almost any point, now moves in such a way as to place the ovoid body and acrosome at the end of the nucleus opposite the condensed chromatin (figs. 29, 30, and 32). The chromatin becomes cylindrical or barrel-shaped, with the nuclear membrane showing to each side, slightly removed from the chromatin, leaving a clear band free from chromatin about the periphery of the nucleus (fig. 35). By this stage the acrosome, which had the form of a finger-like projection, has increased in size and become cone-shaped. It is now about two-thirds the length of the cell and bent to compensate for the central location of its broad end. The acrosome persists at about the same length while the chromatin is elongating and the spiral thread is being formed about the chromatin (figs. 35 to 43).

Soon after the chromatin has reached a length equal to that of the acrosome, a constriction appears on the proximal end of the latter which finally separates a ring-like portion (figs. 46 to 49). Although the chromatin continues to elongate, the length of the acrosome remains about the same.

Because of the extreme length and bent condition of the sperm at this stage, it is necessary to trace a single cell through several sections in order to follow its entire length. By so doing we see that the acrosome begins to thicken and becomes club-shaped (fig. 50), with the ring separating chromatin and acrosome still visible, also a new collar-like region between ring and main part of acrosome. The distal end of the acrosome may be bent to one side, giving it a dipper shape (fig. 51).

Following this stage, the sperm takes the form of a loose coil in which the acrosome retains its club or dipper shape (figs. 52 and 53). The dipper portion of the acrosome becomes pronounced and a crescent-shaped proximal end is differentiated from the original collar and ring portion (figs. 54, 55, and 56). The acrosome may still be seen in the more tightly coiled stage (figs. 57 and 58), but when the mature condition is reached it is no longer visible (figs. 60, 61, and 62). In the

sperm from the female ducts where uncoiling has begun, the acrosome still has the form last noted (figs. 63 and 64).

FORMATION OF SPIRAL THREAD ABOUT CHROMATIN

After taking part in the formation of the acrosome up to the stage when the chromatin is drum-shaped (fig. 35), the Golgi mass now moves up the side of the nucleus, over the end of which it forms a cap (figs. 36 and 37). The ovoid-shaped body of Golgi origin, noted above in figures 29 to 32 and 35, may take part in forming this cap. The nuclear membrane now bulges only where it is not surrounded by the cap (figs. 36 and 41), which fits very tightly, as is seen in cross-section (fig. 38). Soon after it is formed, lines encircling the nucleus appear in the cap (figs. 36 and 37). These lines, which are joined to the projections from the Golgi mass, become double and then spiral (figs. 39, 40, and 41). As the chromatin becomes longer the number of turns increases (figs. 42 and 43). The origin of these threads from the Golgi material is clearly shown in the cells of figures 39 and 42, where the latter has osmiophilic processes which connect with turns of the spiral.

Sokolow ('26) suggests that the chondriosomes in the pseudoscorpion *Chelanops cyrneus* become thread-like, wind about the elongated *Kopfbläschen* (head vesicle of Golgi origin), and fuse at their ends to form the spiral threads. In my preparations the chondriosomes are still spherical after the spiral thread is formed (fig. 43). I also fail to see the head vesicle being punctured by the chromatin in the method which he described in *Chelanops cyrneus*.

The material in the structure similar to his head vesicle, seen in figure 35, may, however, take part in forming the cap about the chromatin as noted above. A body shaped like this is later seen to one side of the acrosome (fig. 42), and if this is identical, could not form a sheath about the chromatin. In the other species studied by Sokolow ('26), *Obisium muscorum*, he is unable to establish the origin of the spiral threads.

Koltzoff ('09) figures, but does not give the origin of, similar spiral threads on the heads of mature sperms of *Monotus* (turbellarian), *Murex brandaris* (snail), *Helix nemoralis* (snail), *Planorbis* (snail), *Paludina vivipara* (snail), *Tritonium corrugatum* (snail), *Lumbricus terrestris* (earthworm), *Formica* (ant), *Opilio* (daddy long legs), *Scyllium canicula* (shark), *Raja clavata* (ray), *Siredon* (salamander), *Coronella* (snake), *Gallus domesticus* (fowl), and *Passer* (sparrow). He found that the spiral threads on the heads of hermit-crab sperms (*Eupagurus prideauxii*, *Munida rugosa*, *Galathea squamifera*, *Pagurus striatus*) originate from chondriosomes (Koltzoff, '06). He suggests that these threads form a skeletal or supporting structure for the nucleus, which he believes would assume a rounded shape if it were not for the confining action of these structures. My observations lead me also to take this view of their function, since before the nuclear membrane was surrounded by the cap or spiral threads it was uniformly separated from the chromatin throughout its length (fig. 35); whereas, after these structures are formed, it bulges only where it is not surrounded by them (figs. 36 to 49).

Although the confining action of the spiral thread about the nucleus is a factor in its elongation and in its decrease in diameter, I believe this change is due in part to the chromatin itself, since the spiral thread is drawn along, is increased in length, and its loops are spread apart in the process (figs. 43, 45, 46 to 50). It may still be seen in late stages of the coiled sperm (fig. 60) and in sperm from the female ducts (fig. 64). The nuclear vesicle retains its position at the tail-bearing end of the chromatin, becoming more flattened and tomato-shaped in some cells (figs. 46 and 49).

While the main mass of Golgi material was at first in contact with the spiral thread throughout its length, it remains near the nuclear vesicle as the chromatin elongates (figs. 42 to 49). In figure 49 many small heavily osmicated granules and small spheres are seen near the remaining Golgi material. Since these granules again have such a position rela-

tive to the Golgi cup in Kolatchev preparations of coiled stages (fig. 53), it is my suggestion that they originate from this cup.

As shown, the axial filament end of the chromatin constantly terminates within such a group of granules. In other less heavily osmicated preparations the cup is clearly seen as a cap in contact with the nuclear wall and surrounded by vesicular chondriosomes (fig. 52). Figure 56 shows an elongated cup of a Flemming smear preparation, about which no granules are preserved. A cup is also visible in late coiled stages (fig. 57). Here we also have a group of small granules, this time near the acrosome, however, while in figure 58 there are heavily osmicated areas which may represent the former granules. A few similar areas in the mature sperm (fig. 62) may represent the final condition of the granules. There is a possibility that the remnant of the main Golgi cup has been extruded in the form of minute globules which surround the encapsulated reproductive cells at this stage. The cup, located near the acrosome in the sperm from the female duct (fig. 63), may be homologous to the above Golgi remnant.

THE CHONDRIOSOMES

In the germinal epithelium the chondriosomes are in the form of heavily osmicated minute vesicles, or groups of vesicles in close contact, located near the nuclei of the syncytium (fig. 1). Those of the early spermatogonia are not grouped so closely together (fig. 1). The arrangement of chondriosomes near the nuclei of the germinal epithelium resembles the grouping of these inclusions to one side of the nucleus in the early spermatogonia; so that it is not difficult to visualize the changing of a portion of the syncytium into the cells of a cyst of definitive germ cells. The appearance of these inclusions depends on the degree of osmication in various preparations; and it is only after studying many sections that one is able to differentiate them from the Golgi material, which the heavily osmicated compact groups in the germinal epithelium closely resemble.

In the early growth period the chondriosome vesicles increase in size somewhat and are grouped among the Golgi particles to one side of the nucleus (fig. 2). The chondriosomes surround the large, lightly osmicating Golgi cap when it is formed in their midst on the same side of the nucleus (fig. 3); however, some of these inclusions may be scattered about the surface of the nucleus. The number of chondriosomes at this stage is much greater than that in the early spermatogonia, while the size of individual inclusions has decreased.

The loops of chromatin seem to converge on the nuclear wall immediately inside the Golgi cap. As the cap moves away from the nuclear surface and becomes spherical, the vesicular chondriosomes increase in size, decrease in number, and are grouped around all sides of this body several layers in thickness (fig. 5). Later they rearrange into a single layer somewhat removed from the sphere, yet enclosing it on all sides like a hull (figs. 6, 7, and 8). Their volume increases, they change from a spherical to a cylindrical shape, with both ends rounded, and the long axis of each chondriosome is directed toward the central sphere (figs. 9 to 11). Figure 12 shows a cross-section of the group. While the two parts of the Golgi material fuse into one mass and this again breaks up, the chondriosomes reassume a spherical shape and become dispersed throughout the cytoplasm (figs. 14 to 16).

During the maturation divisions, the chondriosomes appear as spheres and are dispersed throughout the cell (figs. 17 to 20). They seem scarcely attracted by the force of the division centers in contrast to the Golgi material. There is only an approach to equality in division between the daughter cells. This behavior of chondriosomes is more like that of the scorpions *Opisthocanthus elatus* (Wilson, '16) and *Euscorpium carpathicus* (Sokolow, '13) than like the scorpion *Centurus exilicauda* (Wilson, '16). No chondriosomal ring, such as Wilson ('16) finds in the scorpion *Centurus exilicauda*, is formed in the spermatocyte of this pseudoscorpion. This ring was found to divide during maturation with the precision

of chromosomes. In *Opisthocanthus* and *Euscorpium* there is, as in *Chelanops corticis*, only an approximate segregation of the chondriosomes into the two cells. In the pseudoscorpions *Obisium muscorum* and *Chelanops cyrneus*, Sokolow ('26) also finds that the distribution of chondriosomes between the cells in the maturation divisions is not equal.

In *Chelanops corticis* I find no division of chondriosomes, unless I interpret as such some of the closely grouped chondriosomes in the germinal epithelium (fig. 1), or in the nuclear-cap stage (fig. 3), a stage where there is an increase in number. In the spermatid of a closely related species, *Chelanops oblongus*, I find a division of chondriosomes (fig. 22). These bodies elongate, become dumbbell-shaped, and finally constrict into two daughter chondriosomes. Wilson ('25) finds a similar behavior in the spermatogonia of the scorpion *Opisthocanthus elatus*, and Sokolow ('26) describes this in the growth period of the pseudoscorpion *Chelanops cyrneus* and just before the first maturation division in *Obisium muscorum*.

Following the maturation divisions the chondriosomes become vesicular, clear, and increase slightly in size (figs. 23 and 24). In Kolatchev preparations numerous small, heavily osmicated granules are attached to the chondriosomal surfaces, as mentioned under the Golgi material (figs. 21, 23, and 24). Across the interior of the chondriosomes thin lines are seen which in some cases are correlated to the granules on the surface (fig. 23). Heavier similar lines are also seen in Benda preparations (fig. 34) and in Champy fixation (fig. 25). These lines sometimes form a spiral inside the chondriosomes (figs. 23 and 25). In other preparations the lines are irregular, branching, or parallel (fig. 34).

When the acrosome is just beginning to form, the chondriosomes begin to flatten into disc-shaped bodies (figs. 27 and 28), at which time the threads within are still visible (fig. 27, a Champy preparation). In some Kolatchev preparations near this stage the interior of the chondriosomes appears as two substances, a lightly osmicated material on one side and

a more heavily osmicating material on the other (fig. 26). The chondriosomes flatten until they reach the stage in figure 28, where they are of about the same volume as previously, but with a greater surface exposed to the surrounding cytoplasm. This increase in surface would raise the active mass of the substance of which these bodies are composed, and thus accelerate the reactions in which they could take part. In some chondriosomes one side seems to have a very thin, dark edge (fig. 28, end views of chondriosomes), suggesting that there are two materials present.

By the time the chromatin has begun to condense and contract on one side of the nucleus, the chondriosomes become spherical again (figs. 29 to 31). In Kolatchev preparations they show crescent-shaped or oval, more darkly osmicating bodies on the inside during the stages of rounding (figs. 29 and 30). When the rounded condition has been reached again, there are variations in the contents of the chondriosomes. Some contain a deeply osmicating sphere with one to three granules inside this, others merely a sphere, still others a vesicle enclosing a smaller sphere at an eccentric position (figs. 30 to 32 and 35). We may also have comma-shaped, V-shaped, or question-mark-shaped bodies (fig. 35). The basic chondriosomal substance is quite clear and stands out as a vesicle enclosing these structures.

By the time the Golgi apparatus has formed a cup about the end of the nucleus (fig. 36), the bodies enclosed within the chondriosomes have almost all taken on the form of a small sphere; however, variations may still exist where the spiral thread is well under way (fig. 42). Here we still have within the chondriosomes, small spheres, comma-shaped bodies, and vesicles enclosing a sphere. The heavily osmicating bodies within the chondriosomes are last seen as small vesicles at the stage of elongation of the sperm shown in figure 48.

I know of no other observations of similar heavily osmicating bodies, first on the surface of chondriosomes, then in the form of lines, spheres, granules, and other shapes within the chondriosomes. If these bodies are Golgi material, as I

believe them to be, there is here a very close relationship between this material and the chondriosomes, suggesting that possibly a chemical reaction takes place between the two.

The chondriosomes decrease in size and are gathered into a group, usually at the posterior end of the sperm (figs. 46 to 50). From the center of this group of chondriosomes a thread extends toward the acrosome (figs. 46, 47, and 49). This structure in the stage represented by figure 50 may be distinguished from the elongated chromatin, since it lacks the spiral thread of, and is thicker than, the latter. Although I have no direct evidence, this thread is probably formed from the chondriosomes, since these decrease in size and are not found by me in the mature sperm. Sokolow ('26) also reaches such a conclusion in his work on the pseudoscorpion *Obisium muscorum*.

The chondriosomes are still present as spheres in early stages of the coiling of the sperm (fig. 52), where they surround the Golgi cap. They are not seen by me in the mature sperm (fig. 62).

THE AXIAL FILAMENT

In the spermatid, while the chromatin is condensed at one end of the nucleus, one or more granules are found on the nuclear surface at a point opposite the acrosome (figs. 29, 30, and 32). They are present in an increased number when the Golgi cap is formed over the end of the chromatin (fig. 36). The axial filament first appears in the region of these granules when the spiral thread is double (fig. 40), and since these decrease in number soon after (figs. 42 and 43), I believe that this tail structure takes its origin from them. It is possible that these granules represent part of one or two centrioles, since a bipartite centriole is found in Hemiptera (Payne, '27; Bowen, '22). The two granules which are on the axial filament in figure 46 give further evidence of the probable origin of the axial filament from these bodies.

The axial filament continues to elongate, but is included within the capsule wall which is formed about the sperm

(figs. 54 to 61). This wall is thin at first, but later becomes heavy (fig. 62). The length of the axial filament increases until it is many times that of the chromatin and, in so increasing, the tail structure becomes coiled inside the capsule (figs. 54 to 56).

The capsule becomes smaller in diameter and in so doing draws the chromatin and tail into a tighter coil (figs. 57 and 58). In figure 58, which is a Kolatchev preparation, the gray ring represents the many turns of the axial filament. A cross-section of a similar preparation is shown in figure 59. The capsule takes on an oval shape to which the contents conform (fig. 60). Soon, however, both the loops of chromatin and axial filament twist at the center so as to form a figure-8 shape within the capsule (figs. 61 and 62), which is the mature condition of the sperm.

OTHER CYTOPLASMIC STRUCTURES

In the growth period, immediately following the movement of the cap away from the nucleus, an ovoid-shaped body of an optical refraction similar to that of nucleoli appears in the cytoplasm somewhat removed from the chondriosomal hull (figs. 4 to 6, 9 to 11, 14 to 16). This structure is not Golgi material, since it appears in preparations in which that substance is clearly demonstrated and persists through the time at which the same breaks up (figs. 15 and 16). It is seen in Benda fixations which do not preserve the Golgi material to best advantage (fig. 6). The difference in size and optical refraction distinguishes it from the chondriosomes. I do not find this body during or after the maturation divisions.

In figures 4 and 13, in the growth period, we have flattened, irregular, plate-like bodies on the nuclear surface. These osmicate heavily, but seem to have no relation in function to the Golgi material or chondriosomes. Again in the spermatid there are hemispherical bodies on the nuclear surface which stain much like chondriosomes (figs. 25, 27, and 33). In some cells there is a heavily staining line between the nuclear wall and these bodies, but this may be due merely to greater

adhesion of stain here (fig. 33). In *Chelanops cyrneus* Sokolow ('26) believes that these bodies form a plate over the nuclear membrane at the point where (as he interprets) it is punctured by the elongating chromatin. Figure 44 (of a Benda preparation) gives the appearance of such a plate, but in view of the above-mentioned observations on the formation of a cap and spiral thread about the chromatin, I interpret this ring as merely due to a darker staining of the nuclear membrane on the edge of contact.

In stages of sperm elongation similar to figures 46, 49, and 50, we have one or more small vesicles lying near the chromatin and composed of a material of an optical refraction similar thereto. These bodies migrate along the chromatin to the tail end of the sperm.

I have no suggestions to offer as to the possible function of the above-mentioned inclusions.

FURTHER OBSERVATIONS ON THE MATURE SPERM

As mentioned in connection with the axial filament, the mature sperm is composed of the acrosome, Golgi granules, possibly the chondriosomal thread, the elongated chromatin with its spiral thread, also the axial filament, both of the latter twisted into a 'figure 8.' All of these structures are inside the heavy-walled capsule, which is of an oval shape with flattened sides (fig. 62). In the space about the mature sperm in figure 62 are numerous globules of material, which are not due to precipitation of chemicals, since they appear constantly with various fixing fluids, and are localized in this part of the section. I am not sure of the origin of these bodies, but presume they are some of the extruded cytoplasmic inclusions which I have followed in earlier stages. I find similar globules about the mature sperm of *Chelanops oblongus*, a related species (fig. 65). Sokolow also observes such bodies about the mature sperm of *Chelanops cyrneus* ('26) and in a myriopod, *Polyxenus* ('18), which he regards as *körnige sekrete* (granular secretions).

SPERM IN FEMALE DUCTS

I have been fortunate in sectioning the abdomen of a female specimen which contains both eggs and mature sperm. This individual is of the species *Chelanops oblongus*. The mature sperm of that species differs from *Chelanops corticis* in that the chromatin and axial filament do not twist into a figure 8, but retain their oval shape (fig. 65). In the female ducts I find sperm which are beginning to regain a rounded shape (fig. 63) and others which have attained this condition (fig. 64). The chromatin, coils of axial filament, acrosome, a small sphere, possibly also the Golgi cup, are visible in the capsule. Since the sperm again becomes rounded, as it was before reaching the mature condition, it is probable that it again becomes uncoiled before fertilization takes place. This suggestion is made by Sokolow ('26), but he had been unsuccessful in finding sperm in the female duct.

DISCUSSION

The close proximity and aggregation of the chondriosomes and Golgi material during the growth period, although possibly due to the action of a centrosome, suggest that here an interchange of materials occurs between these bodies. Again in the spermatid, where the small granules of Golgi material are on the surface of the chondriosomes, and then where the various spheres and other inclusions are seen within the latter, there may be an interchange of material in the nature of a chemical reaction.

The active masses of the reacting substances depend upon the total exposed area where reaction might take place. Since for the same volume of material a greater area is exposed in a disc than in a sphere, or in a dispersed condition than when the chondriosomes or Golgi material are in a mass, one would expect a more rapid reaction with these materials under the two former conditions. The changes in shape and distribution of these bodies might thus help regulate metabolism.

There is a striking equality in size and regular arrangement of chondriosomes about the Golgi sphere, which may be

seen in cell after cell in the growth period. This characteristic appearance resembles a group of chromosomes in metaphase plate, and leads one to believe that these bodies have a capacity to limit their size, shape, and position with relation to the Golgi material. The change in shape from a sphere to a disc, and the various spheres, comma-shaped bodies, and granules in their interior during the spermatid stages would further support such a suggestion, also the suggestion that they may have an internal organization which controls their contents.

The chondriosomes are believed by Regaud ('08) and Fauré-Fremiet ('10) to be of a phospholipin nature, with a small amount of protein in combination. Chemical compounds of the first type may characteristically form regular striking 'myelin figures,' and may also form liquid crystals which are easily changed in shape by pressure (Mathews, '25). A good example of such a design is seen in the nebenkern of *Gelastocoris* (Payne, '27). In the pseudoscorpion this property may account for the above-noted equal size and regular arrangement of chondriosomes in the growth period, also the change from a sphere to a disc shape in the spermatid.

I have demonstrated that the Golgi material, in addition to forming the acrosome, gives rise to the spiral thread which encircles the nucleus. Besides having a common origin, both of these structures are associated with the chromatin in the anterior portion of the sperm. The peculiar change in shape of the acrosome in late stages may be associated with the coiled and encapsulated condition of the sperm. The Golgi material in the pseudoscorpion has a far more active reducing action on osmic acid than the chondriosomes have. The Golgi bodies assume shapes which are less regular than those of the chondriosomes. They are more closely attracted than the chondriosomes to the centrosome during the maturation divisions.

Of interest in connection with the encysted, mature sperm is the method of sperm transfer described by Kew ('12) as follows:

The male and female face one another in walking position. The male grasps with one or both hands, one or both hands of the female. . . . At length he extrudes the spermatophore which is attached to the floor in front of the female, where it stands erect or obliquely. From this object the male retires backwards and the female at the same moment comes forward. The movement is just sufficient to bring the female genital opening into contact with the spermatophore and the male product is thus received without delay.

As also suggested by Sokolow ('26), it is presumably to prevent drying during this transfer that the sperm are rolled up into such a compact, immotile form. The uncoiling of the sperm, early stages of which I observe in the female ducts, gives evidence that the encystment is but temporary and serves the above-mentioned purpose.

SUMMARY AND CONCLUSIONS

1. Golgi material is present from the syncytium of germinal epithelium to the mature sperm, where it is represented by acrosome, spiral threads about the elongated chromatin, granules, and undifferentiated remnants.

2. Chondriosomes are present from a similar early stage, seem to take part in forming an elongated thread in the spermatid, and are last seen in the early stages of coiling.

3. During the growth period the Golgi material forms a nuclear cap, which later changes to a sphere, and migrates away from the nucleus. Spherical chondriosomes surrounding the cap become cylindrical about the Golgi sphere.

4. Within the Golgi sphere are one or two darkly staining dots, possibly representing centrioles, but their identity is not proved.

5. The distribution of Golgi material and chondriosomes between the cells in the maturation divisions is only approximately equal. In the case of the chondriosomes this method is in contrast to their equal division in the scorpion *Centrurus exilicauda*.

6. The Golgi material tends to become grouped about the division centers, similar to the behavior in some Hemiptera and in the scorpion *Opisthocanthus elatus*.

7. Within the cytoplasm of the spermatocytes and spermatids are other cytoplasmic inclusions of unknown function.

8. A very long axial filament is formed at the posterior end of the nuclear vesicle from several granules which may represent parts of a centriole.

9. The aggregation of chondriosomes and Golgi material during the growth period, the arrangement of Golgi granules on the chondriosomal surface, and the lines, spheres, and other bodies within the latter in the spermatid suggest that there may be chemical reaction between these inclusions at these stages.

10. The changes in distribution of chondriosomes during the growth period from close aggregation to dispersion, and in shape during the spermatid from sphere to disc, and back to sphere, suggest that there is in these stages a means of partially regulating speed of reactions in which they take part by changing the ratio of their exposed surface to their total volume.

11. The equality in size and regular arrangement of chondriosomes about the Golgi sphere in the growth period, the change in shape from sphere to disc in the spermatid, suggest that these bodies have a form of limiting capacity or internal organization.

12. These regular shapes and arrangements of chondriosomes may be due to their phospholipin nature.

13. The Golgi material in this species has a far more active reducing action on osmic acid than the chondriosomes.

14. The peculiar change in shape, which the acrosome undergoes in the spermatid, is probably associated with the encysted condition of the mature sperm.

15. The chromatin and axial filament are coiled and twisted into a figure 8 within the oval, flat-sided, mature sperm capsule.

16. Within the male reproductive duct, about the mature sperm, are numerous globules which may represent extruded cytoplasmic inclusions.

17. The cause of encystment of the sperm may be explained by the method of sperm transfer as described by Kew.

18. In the female reproductive duct the sperm again uncoils and contains acrosome, chromatin, spiral thread of Golgi origin, axial filament, a sphere, and possibly the Golgi cup.

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EXPLANATION OF PLATES

All figures have been drawn to the same scale. A 2-mm. objective and a Leitz no. 4-b eyepiece were used. The camera-lucida drawing was enlarged to twice the original. The final drawing was reduced to two thirds. Magnification on the printed page, $\times 2400$. All figures are of *Chelanops corticis* unless otherwise indicated.

ABBREVIATIONS

<i>A.</i> , acrosome	<i>N.</i> , nucleus
<i>A.F.</i> , axial filament	<i>N.V.</i> , nuclear vesicle
<i>A.R.</i> , acrosomal ring	<i>O.B.</i> , ovoid body
<i>C.</i> , centriole	<i>O.G.</i> , ovoid body of Golgi origin
<i>Ch.</i> , chromosomes	<i>P.C.A.</i> , proximal crescent-shaped part of acrosome
<i>C.W.</i> , capsule wall	<i>P.N.</i> , plate-like bodies on nuclear surface
<i>E.I.</i> , extruded cytoplasmic inclusions	<i>R.</i> , ring at junction of nuclear vesicle and Golgi cap
<i>E.S.</i> , early spermatogonia	<i>S.T.</i> , spiral thread of Golgi origin
<i>G.</i> , Golgi material	<i>V.</i> , vesicles migrating along elongating sperm
<i>G.C.</i> , Golgi cap	
<i>H.B.</i> , hemispherical bodies on nuclear surface	
<i>M.</i> , chondriosomes	
<i>M.T.</i> , chondriosomal thread	

PLATE 1

EXPLANATION OF FIGURES

1 Germinal epithelium with enclosed cyst of early spermatogonia. Vesicular chondriosomes grouped near nuclei in both stages. Golgi material of germinal epithelium takes three forms, all more diffuse than in spermatogonia. Kolatchev.

2 The beginning of the growth period. Mass of chondriosomes and Golgi granules chiefly at one side of nucleus. Kolatchev.

3 Chondriosomes aggregated about Golgi cap, which is located on the nuclear surface. Kolatchev.

4 Chondriosomes about Golgi cap which has become spherical and removed from nucleus. Ovoid body first appears in cytoplasm and plate-like bodies on nuclear surface. Champy (without mordant).

5 Golgi sphere shows heavily osmicated granules. Part of Golgi material outside chondriosomal hull. Kolatchev.

6 Chondriosomes form single layer around Golgi sphere on all sides. Benda.

7 and 8 Sectional views of Golgi sphere and chondriosomal hull similar to that in figure 6. One or two darkly staining particles inside sphere. Figure 7, Benda; 8, Flemming.

9 Chondriosomes become cylindrical. Proximity to Golgi material and arrangement suggest chemical interchange. Kolatchev.

10 Golgi granules scattered through cytoplasm and migrating between chondriosomes of hull to central Golgi sphere. Single large Golgi mass outside chondriosomal hull. Kolatchev.

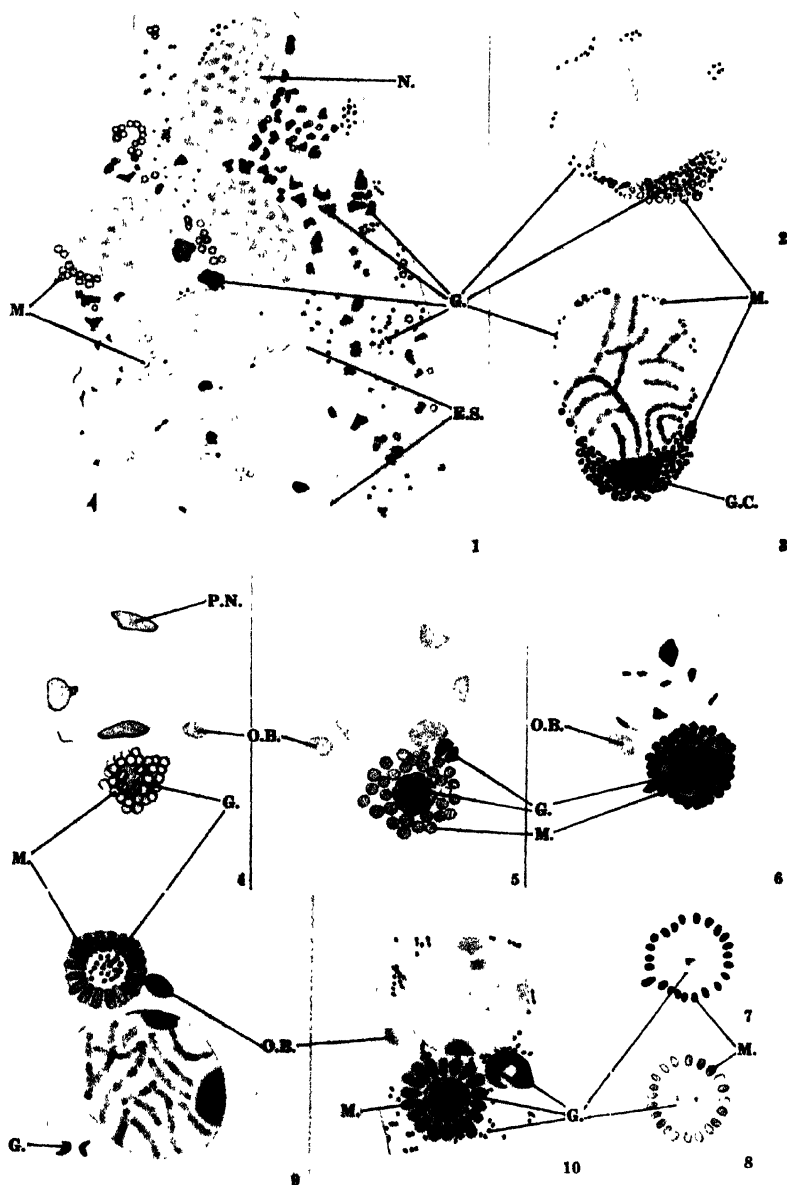


PLATE 2

EXPLANATION OF FIGURES

11 Cylindrical chondriosomes on all sides of Golgi sphere. Parts of Golgi material outside hull. Benda. *Chelanops oblongus*.

12 Sectional view of chondriosomal hull and Golgi sphere of figure 11. Benda. *Chelanops oblongus*.

13 Apparent thread-like flowing of outer Golgi material between chondriosomes to inner Golgi sphere. Plate-like bodies on nucleus. Mann-Kopsch. *Chelanops oblongus*.

14 Fusion of all Golgi material previous to fragmentation as in figures 15 and 16. Chondriosomes resume spherical shape and scatter as Golgi fusion takes place. Kolatchev.

15 Golgi material fragmented, chondriosomes dispersed previous to maturation divisions. Ovoid body last visible.

16 Fragmentation of Golgi material into rod-like units. Chondriosomes smaller in this species. Mann-Kopsch. *Chelanops oblongus*.

17 and 18 Maturation division. Chondriosomes dispersed. Division of chondriosomes and Golgi material between resulting cells only approximately equal in contrast to equal division in true scorpion. Mann-Kopsch. *Chelanops oblongus*.

19 and 20 Maturation division. Golgi material grouped about poles. Chondriosomes larger than in species of figures 17 and 18. Kolatchev.

21 Spermatid with reassembled large Golgi cup, also Golgi granules dispersed in cytoplasm and on surfaces of vesicular chondriosomes. Kolatchev.

22 Spermatid with various stages in division of chondriosomes. Flemming. *Chelanops oblongus*.

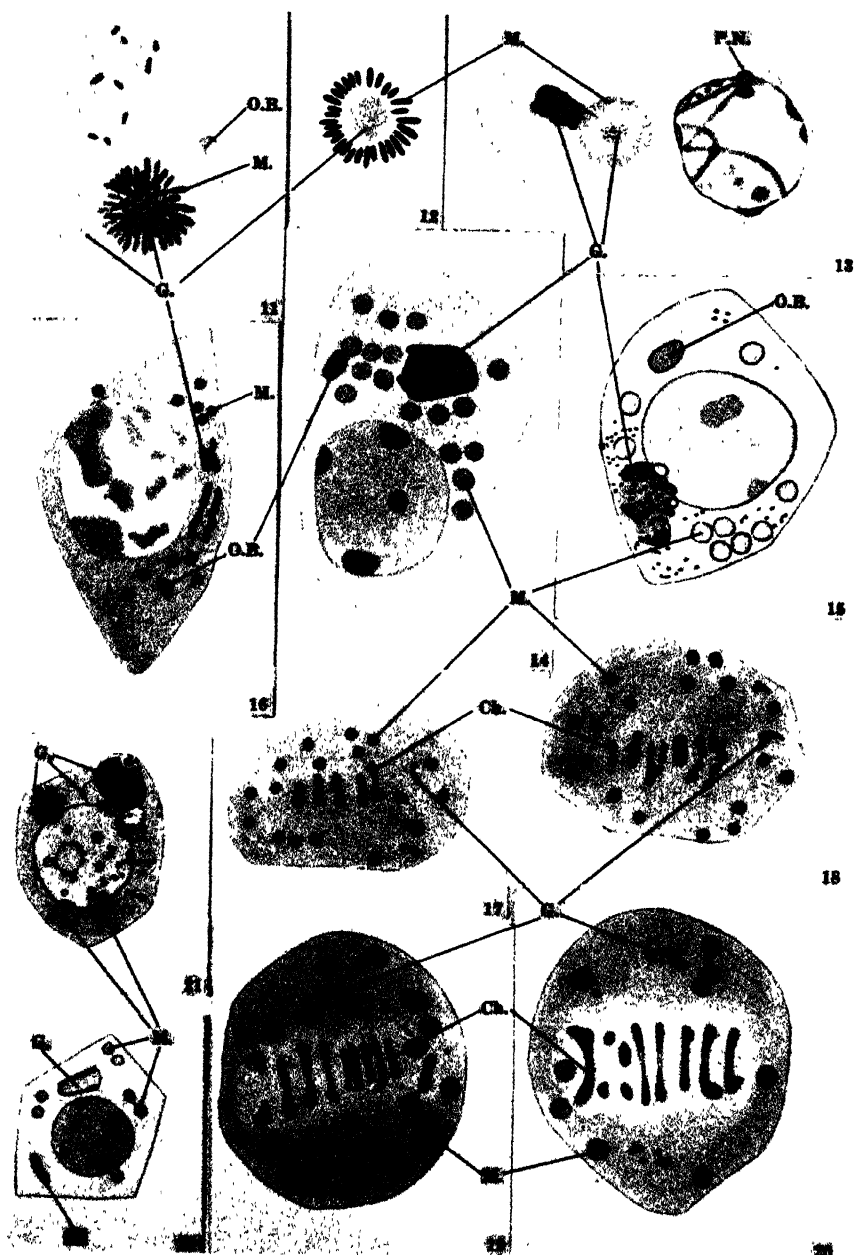


PLATE 3

EXPLANATION OF FIGURES

23 and 24 Early spermatid. Golgi material reuniting to form large mass. Golgi granules on chondriosome surfaces seem correlated to lines within, suggesting possible chemical interchange. Kolatchev.

25 Lines within chondriosomes heavier due to fixation. Hemispherical bodies on nuclear surface. Champy (without mordant).

26 Early stage in formation of acrosome from Golgi cup. Chondriosomes show duplex make-up. Kolatchev.

27 Chondriosomes begin to flatten, a possible means of regulating chemical reaction by increasing surface. Champy (without mordant).

28 Acrosome increases. Chondriosomes further flattened to discs. Golgi material partly as cup, partly as granules in cytoplasm. Kolatchev.

29 Later spermatid. Ovoid body of Golgi origin between Golgi cup and acrosome. Chromatin condensed. Chondriosomes again becoming spherical. Centrosomes on nuclear surface. Kolatchev.

30 to 32 Hollow and solid spheres, also comma-shaped bodies within chondriosomes, possibly of Golgi origin, indicate close relationship of these inclusions. Golgi granules surround acrosome of figure 32. Kolatchev.

33 Early spermatid. Hemispherical bodies on nuclear surface. Dark-staining area of contact. Benda. Chelanops (new species related to tumidus).

34 Early spermatid. Lines within chondriosomes single or forked. Fixation and species as in figure 33.

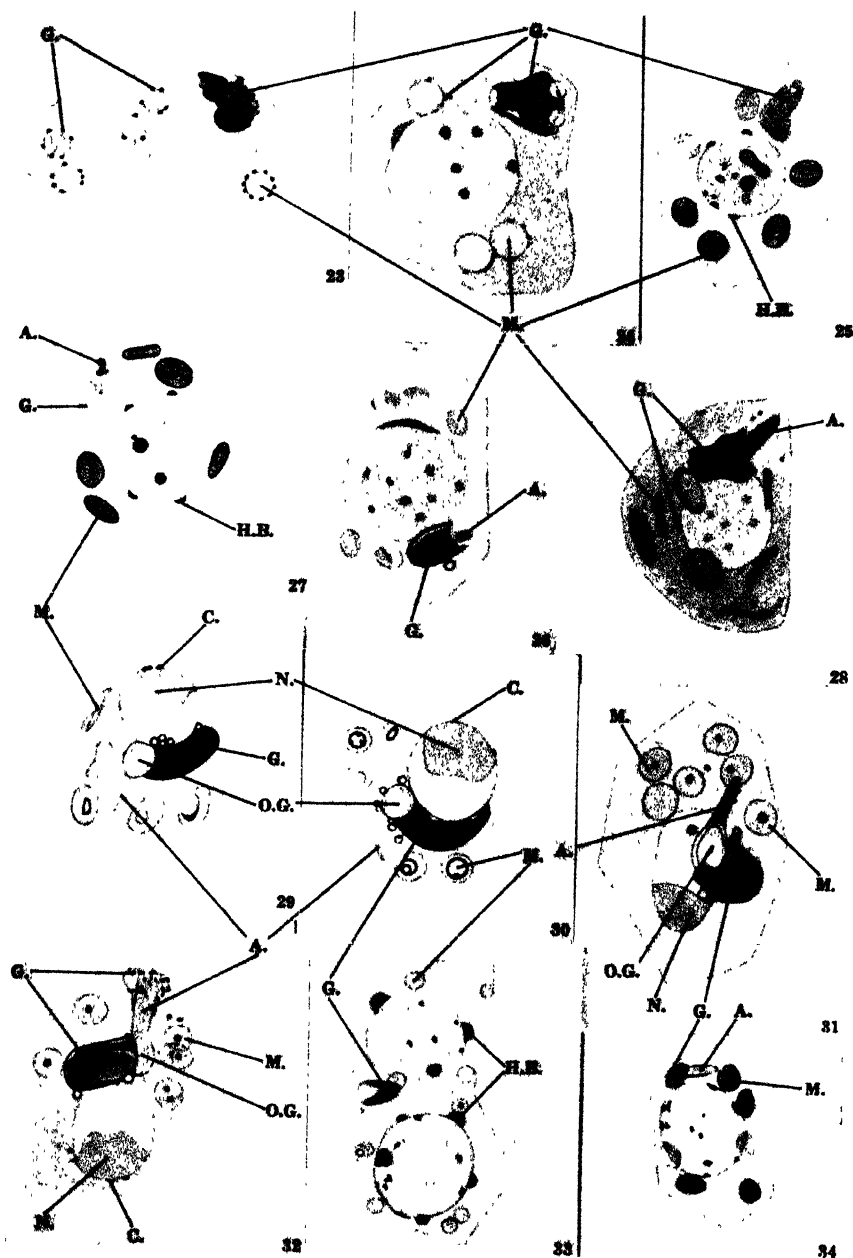


PLATE 4

EXPLANATION OF FIGURES

35 Later spermatid. Acrosome longer. Chromatin rearranged within nuclear wall. Chondriosomes with various inclusions. Kolatchev.

36 and 37 Golgi mass forms cap constricting nuclear membrane. Lines appear in Golgi cap. Centrioles at opposite end of nucleus. Kolatchev.

38 Cross-section of stage similar to figure 37, showing relationship of Golgi mass, cap, and nucleus. Kolatchev.

39 to 43 Spiral threads about nucleus formed from Golgi mass. Nucleus elongates. Centrioles give rise to axial filament. Ovoid body of figure 32 last seen. Kolatchev.

44 Benda fixation shows dark-staining ring between Golgi cap and nuclear wall. Chelanops (new species related to *tumidus*).

45 Nucleus further elongates, spreading turns of Golgi spiral thread. Thick thread originates among chondriosomes. Kolatchev.

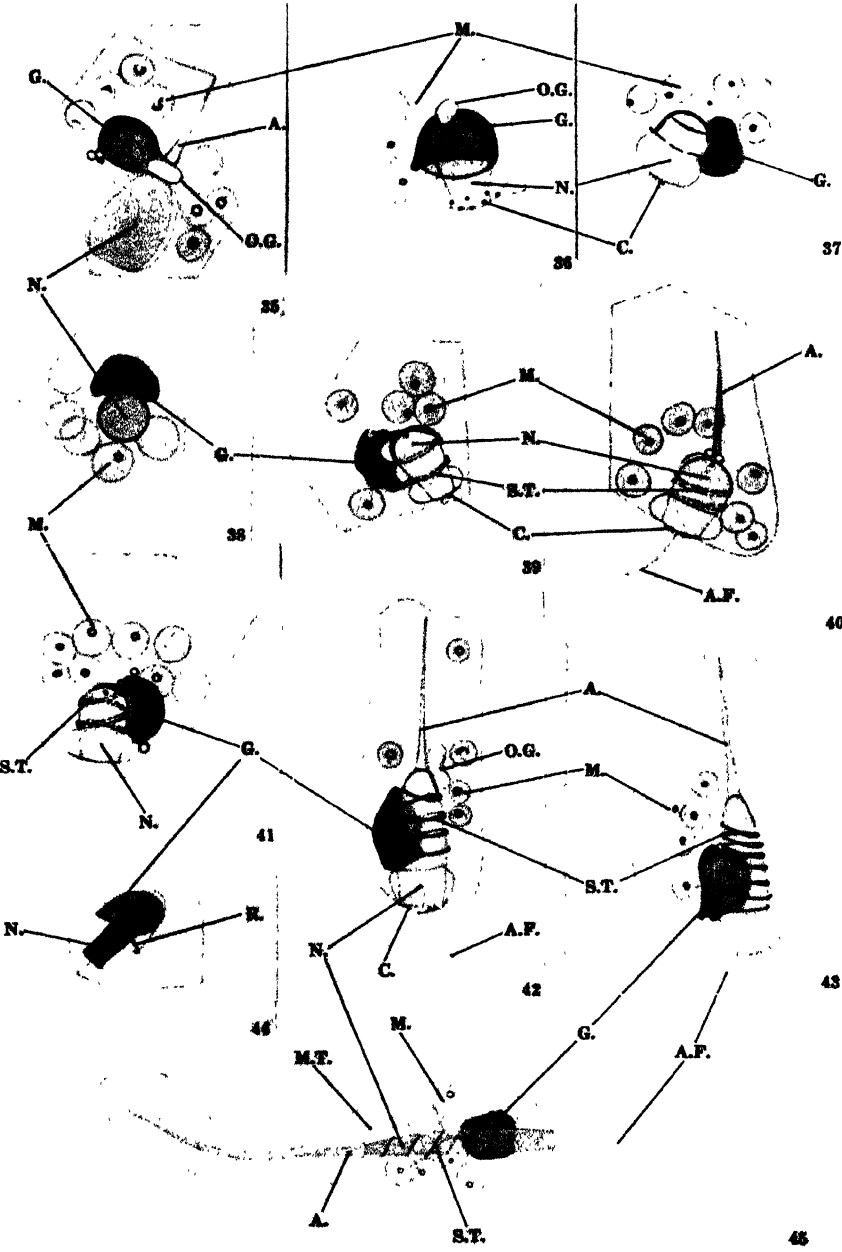


PLATE 5

EXPLANATION OF FIGURES

46 to 49 Stages in further elongation of sperm. Chondriosomal thread increases in length. Ring forms at proximal end of acrosome. Spheres within chondriosomes last seen in figure 48. Figure 46, Champy with pyroligneous-chromic-bichromate mordant; figure 47, Benda. Both *Chelanops oblongus*. Figures 48 and 49, Kolatchev.

50 Acrosome changes from cone to club shape. Acrosomal ring remains at proximal end. Champy (without mordant).

51 Further change in distal end of acrosome to dipper shape. Champy (without mordant).

52 and 53 Early bending of sperm in preparation for coiling. Chondriosomal thread bends with nuclear thread. Kolatchev.

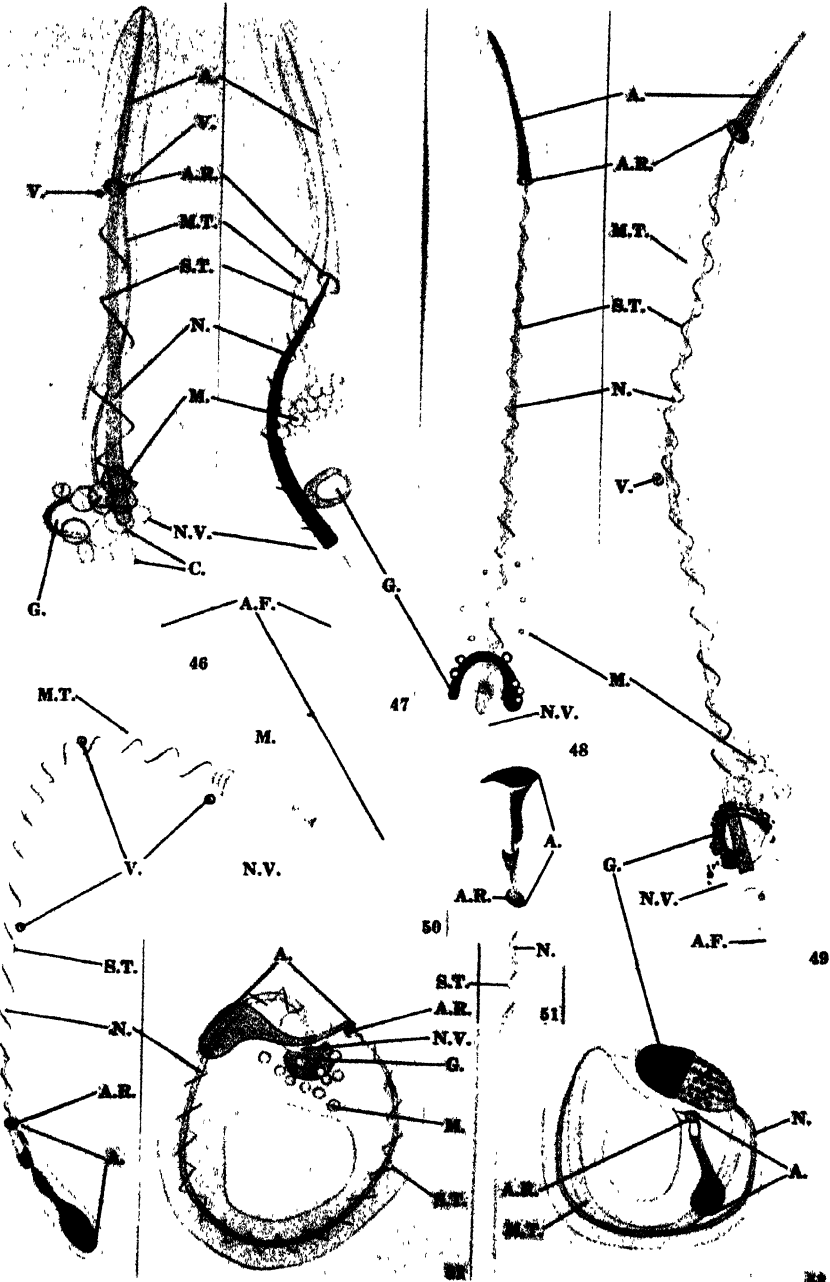


PLATE 6

EXPLANATION OF FIGURES

54 to 56 Acrosome further differentiated. Axial filament much elongated, coiled, and enclosed within capsule wall. Flemming smear.

57 and 58 Capsule wall tighter. Acrosome still visible. Figure 57, Flemming smear; figure 58, Kolatchev.

59 Cross-section through stage similar to figure 58. Coils of nuclear thread parallel with axial-filament coil. Kolatchev.

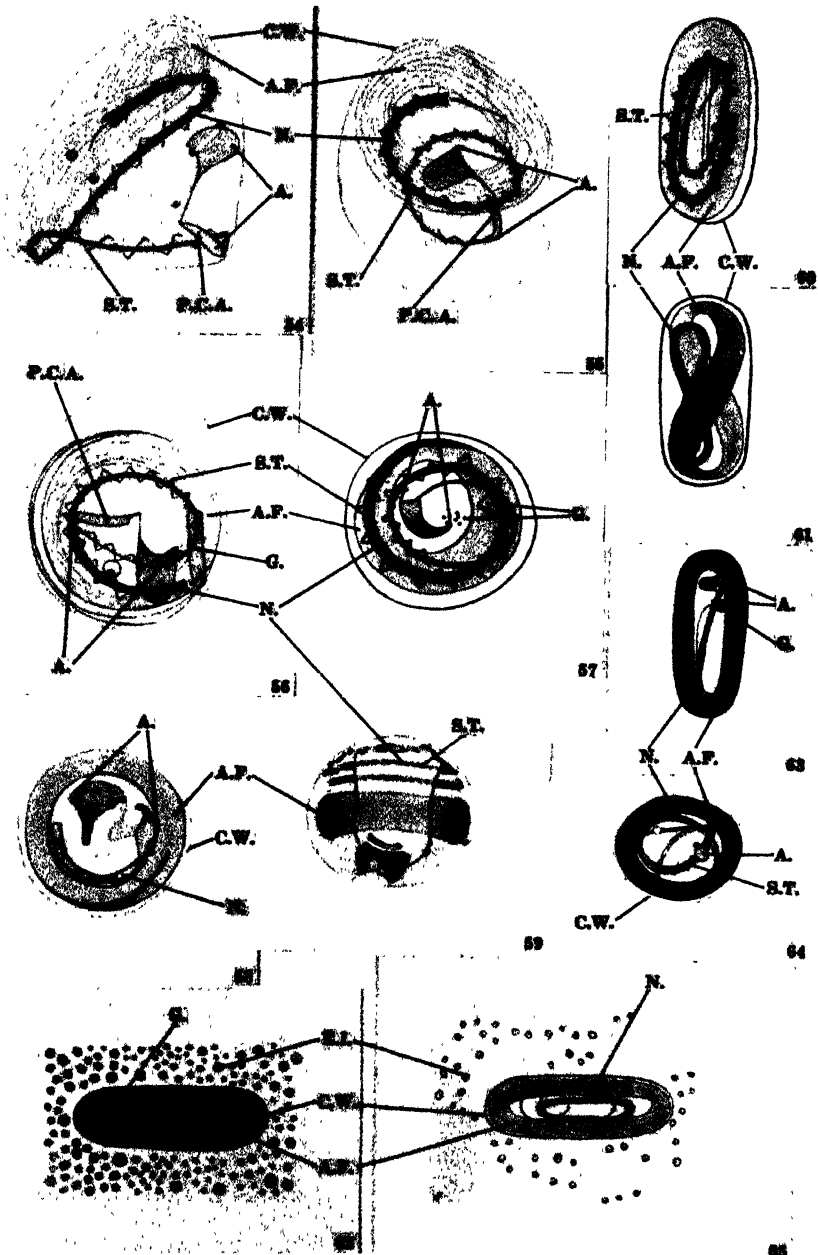
60 Capsule takes oval shape. Spiral thread persists on nucleus. Flemming smear.

61 Nuclear thread and axial-filament coils twisted into figure 8. Flemming smear.

62 Mature sperm, with Golgi remnants and heavy capsule wall, surrounded by extruded cytoplasmic inclusions. Kolatchev.

63 and 64 Sperm of *Chelanops oblongus* uncoiling within female duct. Acrosome, Golgi cup, nucleus, axial filament still present. Benda.

65 Mature sperm, with capsule, of *Chelanops oblongus* surrounded by extruded inclusions. Champy with pyroligneous-chromic-bichromate mordant.



THE EARLY DEVELOPMENT OF THE DUCK'S EGG, WITH SPECIAL REFERENCE TO THE ORIGIN OF THE PRIMITIVE STREAK

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FOUR PLATES (TWENTY-SIX FIGURES)

AUTHOR'S ABSTRACT

This study deals with the development of the duck embryo from the unincubated blastoderm to the 1-to-3-somite stage. In the unincubated stage the thickened posterior germ wall is the most active region mitotically, contributing cells to complete the ectodermal layer. The slightly thickened ectoderm of the posterior half of the pellucid area is the precursor of the embryonic shield. During incubation this ectoderm increases in height, forming the embryonic shield. The primitive streak arises in the posterior part of the shield, where tall ectodermal cells in the midline become several-layered and proliferate cells from their ventral surface which migrate laterally, becoming mesoderm. At first short and broad, the streak becomes longer and narrower until it attains its definitive stage.

The greatest proliferation of ectodermal cells occurs always at the anterior end of the streak of all stages, forming at the definitive stage a definite thickening—Hensen's node. Proliferation diminishes posteriorly along the streak.

The number and orientation of mitotic figures indicate cell movements as follows: Prestreak stages—ectodermal cells migrate from posterior part of shield toward midline, thence forward in midline, early streak stages—anterior movement in streak and to sides of streak, and lateromedial movement from sides toward middle of streak; definitive streak stages—postero-anterior and lateromedial migration of cells.

The embryonic axis, of prestreak and streak stages, lies approximately at right angles to the egg axis in slightly over 50 per cent of the cases.

INTRODUCTION

The early development of the bird egg has been the object of but few investigations, most of which concern the chick. An accurate and complete knowledge of the morphology of these early stages and the mode of formation of the primitive streak from the unincubated blastoderm has become essential for studies in experimental embryology of the bird. A careful and detailed study has therefore been made of the development of the duck embryo from the unincubated blastoderm to the one-to-three-somite stage, including a study of the orientation of mitotic figures at various stages as indicators of the direction of growth and cell movements. The results of this investigation form the subject of the present paper. Pertinent literature will be considered in connection with the various topics.

The problem was suggested by Dr. B. H. Willier, to whom I wish to express my sincere thanks for his advice, suggestions, criticisms, and aid during the progress of the work. I am deeply indebted to Dr. L. H. Hyman for aid in editing the paper.

MATERIAL AND METHODS

White Pekin duck eggs were used, obtained from a farm in Indiana, transported by express to Chicago, requiring about twenty-four hours for transit. The exact time of laying was unknown. The eggs were obtained from March through July and gave a high percentage of hatch. About 14 per cent were sterile and a small number died during incubation. From over 1200 eggs used, 852 embryos were secured, of which 208 were mounted whole, 212 sectioned, and 82 others studied as cleared specimens in wintergreen oil. The remaining embryos have been preserved either in wintergreen oil or embedded in paraffin, but have not been studied.

To insure a uniform rate of development from different lots of eggs, great care was exercised to keep the temperature of the incubator constantly at 39°C. Whenever eggs were put into the incubator, from 300 to 500 cc. of water were added to the sand tray. The time of placing the eggs in the incubator and of removing them was carefully recorded.

The blastoderm was removed as usual under warm salt solution. To aid in separating the vitelline membrane a few drops of a modified Mayer's hardening solution (5 cc. nitric acid, 95 cc. water, two drops glycerin) were added to the blastoderm in a watch-glass. The addition of glycerin to the formula keeps the blastoderm smooth and prevents too great hardening. The vitelline membrane was removed with a wet camel's-hair brush of just a few hairs, and the blastoderm transferred to clear warm salt solution, after which it was fixed for one-half to two hours in Kleinenberg's picro-sulphuric fluid, modified in accord with Lillie's suggestion by the addition of 2.5 to 5 per cent of glacial acetic acid, depending on the size of the embryo. After fixation, the embryos

were washed in weak alcohol and run up to 70 per cent, in which they were preserved.

To study the relation of embryonic axis to the egg axis, the egg was candled and the position of the blastoderm marked on the shell. With the blunt end of the egg to the operator's left, the egg was opened at the marked place, and a piece of agar plate stained with neutral red applied for a few seconds to the vitelline membrane over the blastoderm. The embryo stained by the neutral red is clearly visible, and its angle with the egg axis was then determined with the aid of a protractor in which a triangular opening whose apex pointed toward the 90° mark had been cut. With this opening placed over the blastoderm and the protractor put in line with the egg axis, the deviation of the embryonic axis from the perpendicular could be determined.

Alum-cochineal or Ehrlich's haematoxylin was used for most of the whole mounts and sections. Some serial sections were stained with iron haematoxylin. Mayer's haemalum was advantageous for the study of mitotic figures which were counted under oil immersion. Sections were cut 5 μ and 6 μ in thickness. Transverse sections were prepared of all stages and sagittal sections of many stages in addition. Various measurements were made with an ocular micrometer.

RELATION OF THE EMBRYONIC AXIS TO THE EGG AXIS: BILATERALITY

That a relation exists between the embryonic axis and the egg axis in the hen was discovered by von Baer (1828), who stated that when the blunt end of the egg is held to the observer's left, the embryonic axis lies at right angles to the egg axis with the head of the embryo directed away from the observer. Later investigators found a variable amount of deviation from this rule. Dalton ('81) reported exact agreement with the rule in 50 per cent of cases and Duval ('84) in 76 per cent, for the hen. In the emu Haswell ('87) found that the embryo is usually at right angles, not infrequently oblique, but never parallel, to the egg axis. Féré

('96, '97) recorded a deviation of 45° or more from the perpendicular in 10 to 30 per cent of hen's eggs and in 65 per cent of duck's eggs, the latter bird being therefore more variable than the former. According to Rabaud ('08), the embryonic axis was exactly perpendicular to the egg axis in only nine out of one hundred hen's eggs, but in 76 per cent the axis varied less than 45° to the right and left of the perpendicular. For the pigeon, Patterson ('09) reported that the embryonic axis makes an angle of 45° with the egg axis in 90 per cent of cases, while Bartelmez ('18) found the angle to lie between 45° and 90° in 85 per cent of cases, with variations to almost 180° . The variations are less in eggs from a single bird. The latest data on the hen's egg are those of Kopsch ('27), who found that in only eleven out of 155 eggs was the embryonic axis at right angles to the egg axis, but in fifty cases it was approximately so and ninety-seven, or over 60 per cent, varied 45° or less from the perpendicular in both directions. Kopsch's data agree very well with those of Rabaud, and it may be concluded that for the hen's egg, the embryonic axis is exactly or nearly at right angles to the egg axis in only a small percentage of cases (less than 10 per cent), but varies 45° or less to right and left from the position in 60 to 75 per cent of the embryos.

In the course of my study of the development of the duck, considerable data were accumulated on the orientation of the embryonic axis in unincubated eggs, incubated eggs before the appearance of the primitive streak, and primitive-streak stages. In all cases the blastoderm was rendered visible by applying a neutral-red agar plate to it in situ. In unincubated eggs a deeply stained crescentic area appears at the posterior edge of the area pellucida; in incubated blastoderms in prestreak stages, an ectodermal thickening, the so-called embryonic shield, occupies the posterior half of the pellucid area. A line bisecting these areas was considered to represent the embryonic axis. The data on thirty-one unincubated eggs and 120 eggs incubated one to twelve hours, stages prior to the appearance of the primitive streak, are

summarized in table 1. Of these eggs, the embryonic axis was exactly at right angles to the egg axis in forty-one cases, or 27 per cent. The angle lay within 15° to the right of the perpendicular position in sixteen cases and to the left in twenty-five cases, making a total of eighty-two cases, or over 54 per cent, in which the axis is approximately perpendicular to the egg axis. There were thirty-one additional cases where the axis lay between 15° and 45° to the right of the perpendicular and fourteen to the left. Thus there was a total of 127 embryos out of 151, or over 84 per cent, where the embryonic axis lay within 45° to the right or left of, or on the perpendicular with, the egg axis. This gives a higher percentage of approximate agreement with von Baer's rule for the duck than for the hen. Of the remaining cases, the deviation lay between 0° and 45° in ten cases and 135° and 180° in eleven embryos. There were three inversions where the anterior end of the axis was directed toward the observer when the egg was held in the stated position.

The data for the primitive-streak stages, usually incubated more than nine hours, are presented in table 2. There were 174 embryos, of which the axis lay in the perpendicular in forty-five cases, or approximately 26 per cent. Deviations within 15° of the perpendicular amounted to eighteen to the right, or over 10 per cent, and thirty to the left, or over 17 per cent; the total number approximating the perpendicular is thus ninety-three, or over 53 per cent. Axes lying between 15° and 45° to the right of the perpendicular totaled thirty-one and to the left, sixteen. On adding these forty-seven cases to the preceding, it is seen that 140 cases, or over 80 per cent, of primitive streaks lie within 45° of the perpendicular with the egg axis. The remaining streaks were orientated more than 45° from the perpendicular—seventeen to the right and thirteen to the left—plus four inversions.

The data for the early and late stages are in very good agreement and indicate that the angle of the axis does not alter during development. The agreement with von Baer's rule appears to be somewhat better for the duck than for the

chick. In the duck the embryonic axis was found to lie at right angles to the egg axis in over 26 per cent of cases, as compared with less than 10 per cent in the chick, and to approximate this position in more than half of the specimens, as compared with one-third agreement in the chick. Devia-

TABLE 1

Relation of the embryonic axis to the egg axis before the appearance of the primitive streak

DEGREES	RELATION OF EMBRYONIC AXIS TO EGG AXIS	NUMBER OF EGGS	PERCENTAGE
90	Embryonic axis perpendicular to egg axis	41	27
76-89	Embryonic axis within 15° to the right of the perpendicular	16	11
91-103	Embryonic axis within 15° to the left of the perpendicular	25	16.5
46-55	Embryonic axis within 15° to 45° to the right of the perpendicular	5	3.3
56-65		8	5.2
66-75		18	12.0
106-115	Embryonic axis within 15° to 45° to the left of the perpendicular	12	8.0
116-125			
126-135		2	1.3
0-5	Embryonic axis within region from 0° to 45° from the horizontal, i.e., the egg axis	1	0.7
10-25		3	1.9
26-35		2	1.3
36-45		4	2.6
146-155	Embryonic axis within the region from 135° to 180° from the horizontal	4	2.6
156-165		3	1.9
166-175		2	1.3
176-180		2	1.3
Inversion		3	1.9

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tions of less than 45° from the perpendicular include over 80 per cent of duck embryos, as contrasted with less than 75 per cent in the chick. The results do not agree with Féré's ('96) findings for the duck and indicate a less rather than a greater variability of the position of the axis in this bird than in the chick. A general impression was gained during the investigation that deviations are more common to the right than to

the left, but the figures lend only a slight support to this conclusion. However, Kopsch's data on the chick show a decided tendency toward right deviation.

This study has shown that regardless of the angle between the embryonic axis, the blastoderm itself is bilaterally sym-

TABLE 2

Relation of embryonic axis to egg axis in primitive-streak stages

DEGREES	RELATION OF EMBRYONIC AXIS TO EGG AXIS	NUMBER OF EGGS	PERCENTAGE
90	Embryonic axis perpendicular to egg axis	45	25.9
76-89	Embryonic axis within 15° to the right of the perpendicular	18	10.3
91-105	Embryonic axis within 15° to the left of the perpendicular	30	17.2
46-55	Embryonic axis within 15° to 45° to the right of the perpendicular	5	2.9
56-65		4	2.3
66-75		22	12.6
106-115	Embryonic axis within 15° to 45° to the left of the perpendicular	7	4.0
116-125		6	3.4
126-135		3	1.7
0-5	Embryonic axis within region from 0° to 45° from the horizontal, i.e., the egg axis	3	1.7
10-25		5	2.9
26-35		4	2.3
36-45		5	2.9
136-145	Embryonic axis within the region from 135° to 180° from the horizontal	2	1.2
146-155		2	1.2
166-175		4	2.3
176-180		5	2.9
Inversion		4	2.3
		174	

metrical even before incubation. The crescentic area which is seen after staining at the posterior edge of the area pellucida is bilateral and a line bisecting it marks the future axis of the embryo. Bilaterality is thus fully established before the organization of the primitive streak, which forms upon an already existing axis of symmetry and does not itself determine the axiation or bilaterality of the embryo.

STRUCTURE OF THE UNINCUBATED BLASTODERM

The unincubated blastoderm of the chick has been repeatedly described in the older literature, but here it will suffice to combine Lillie's ('19) and Wetzel's ('29) descriptions for comparison with the duck. The unincubated blastoderm of the hen's egg averages 4.4 mm. in diameter (Edwards, '02), of which 1.75 to 2.5 mm. is occupied by the central transparent area pellucida (Keimfeld of Wetzel), the remainder by the darker peripheral area opaca. Between the area pellucida and the yolk is the subgerminal cavity (subgerminale Höhle or Dotterhöhle of Wetzel), bounded peripherally by the germ wall, a thick layer of cells continuous with the yolk. The germ wall is frequently syncytial and filled with yolk inclusions. It is thicker at the future posterior end of the blastoderm than elsewhere. The area pellucida consists of an outer ectoderm, a single layer of cylindrical cells, and an inner incomplete entoderm. The ectoderm is continuous peripherally with the germ wall. The entoderm underlies only the posterior part of the area pellucida and is there continuous with the thickened germ wall. Anteriorly and laterally the entoderm does not reach the germ wall, but terminates with irregular edges. The ectoderm is thickened in the central region of the area pellucida, constituting the embryonic shield. The entoderm, elsewhere one-layered, is several-layered centrally, forming a thickening called by Wetzel the Entodermhof.

The unincubated blastoderm of the duck has not been hitherto described; it corresponds closely to that of the chick. In stained whole mounts it is seen as a circular area divisible into the central transparent area pellucida and the opaque peripheral area opaca. Immediately bounding the area pellucida is the germ wall, notably thicker and wider at the future posterior end of the blastoderm. The diameters of thirty-seven unincubated blastoderms mounted in balsam or oil of wintergreen ranged from 1.8 to 2.8 mm., with an average of 2.24 mm.; those of the area pellucida, from 0.83 mm. to 1.5 mm., with an average of 1.14 mm. Thus the duck ger-

minal disk is smaller than that of the chick despite the larger size of the egg. Figure 1 is a photograph of the unincubated blastoderm of the duck.

Study of sections of twelve unincubated blastoderms shows that in all cases they are composed of two layers, ectoderm and entoderm. The ectoderm is a single coherent layer of epithelial cells throughout the areas pellucida and opaca. In the posterior half of the pellucid area, the ectoderm is slightly thicker than elsewhere, composed of tall, cylindrical cells (average height, 25 μ), with irregularly arranged nuclei, not lying in one level. The thickened ectoderm is probably the precursor of the embryonic shield. Elsewhere in the area pellucida the ectoderm is lower, of somewhat hexagonal cells. It extends out over the area opaca as a still more flattened membrane, reaching beyond the germ wall, particularly posteriorly and laterally. The ectoderm cells have definite cell walls, distinct round nuclei, and a moderate number of yolk granules.

The entoderm underlies the whole area pellucida, but is not a coherent layer. In the posterior part of the area pellucida it is several layers thick and is closely united with the germ wall. Anteriorly from this region, the entoderm thins rapidly and is soon reduced to a one-celled condition, where, however, the cells are very loose and incoherent. In fact, in the anterior part of the area pellucida, the entoderm consists of scattered single cells, with here and there groups of a few cells. The entoderm cells are spherical and contain numerous yolk inclusions; and many yolk bodies are intermingled with them. The entoderm touches the germ wall throughout the entire circumference of the germ wall, but is closely related to it only posteriorly.

Encircling the area pellucida is a thick zone which is wider and thicker posteriorly than elsewhere. This zone I have taken to include the germ wall, zone of junction, and margin of overgrowth of Lillie's ('19) and Patterson's ('09) descriptions. It is not possible in the duck to distinguish between a central cellular part of the germ wall and a peripheral syncy-

tium (zone of junction). Consequently the entire thickening will be designated germ wall. It is almost twice as thick at the posterior as at the anterior border of the area pellucida. The average thickness for twelve cases is $83.5\ \mu$ posteriorly, 42 to $30\ \mu$ at the sides, and $42\ \mu$ anteriorly. The germ wall consists of large cells without distinct boundaries and hence forming a syncytium. This syncytium is packed with yolk spherules, especially where it borders the entoderm, and is continuous below with the yolk. Peripherally beyond the thickened germ wall, the ectoderm extends out over the free yolk as a flattened epithelium, distinct from the yolk. This may correspond to the margin of overgrowth of Lillie and Patterson, but does not quite fit their figures and definition. In nine of the twelve blastoderms, this free ectodermal margin is well developed; in the other three the ectoderm does not extend beyond the germ wall thickening.

A median sagittal section through the unincubated blastoderm is shown in figure 9. Each end of the section is composed of the thick germ wall, which is much thicker and broader at the posterior end. It begins posteriorly as a pointed projection of rather well-defined epithelial cells, which may be termed the margin of overgrowth. Just anterior to this is the thick germ wall proper, whose upper layers are epithelial with distinct cell walls, lower layers more or less syncytial with numerous yolk inclusions and intermingled with free yolk. Anterior to the germ wall is the area pellucida, consisting of a continuous ectoderm above, composed of rather irregularly shaped and arranged cells, and an incoherent entoderm below of rounded, separate yolk-laden cells. The entoderm is three or four layers thick immediately in front of the posterior germ wall, with which it is continuous. Anteriorly it thins to a single layer of separate cells which here and there may cohere for a short distance. The ectoderm of the posterior half of the area pellucida is thicker than that of the anterior half. The anterior germ wall is similar to the posterior, but is less thick. The entoderm reaches the anterior germ wall. In this particular

blastoderm the free marginal overgrowth of the ectoderm mentioned above is lacking.

The number and orientation of mitotic figures were carefully studied in sections of nine unincubated blastoderms. Three orientations were considered: dorsoventral spindles, oriented at right angles to the surface; anteroposterior spindles, oriented parallel to the surface, but lying on lines parallel to the anteroposterior axis of the blastoderm; and lateral spindles, also horizontal and parallel to the surface, but lying on lines crossing the blastoderm at right angles to its anteroposterior axis. In each blastoderm the mitotic figures were counted in one section each of three different levels. For the unincubated blastoderm the levels chosen were the posterior thickening of the germ wall, the posterior part of the area pellucida, and the central part of the area pellucida. But one section was counted for each blastoderm and those of the same level were added together for the nine blastoderms. The figures given are thus the sum of the mitoses found in single corresponding sections of nine blastoderms. For the posterior thickening of the germ wall, the entire count thus obtained for the nine blastoderms, one section of each, was fifty-four dorsoventral spindles, sixty-one anteroposterior, and twenty-three lateral spindles. It is assumed that the dorsoventral spindles are adding cells to the thickness of the germ wall, the anteroposterior spindles proliferating cells into the entoderm, and the third class helping increase the circumference of the germ wall. In cross-sections of the posterior part of the area pellucida, including laterally the germ wall, were counted a total of eighteen dorsoventral, forty-two anteroposterior, and twenty-eight lateral mitotic figures in the ectoderm; for the entoderm the figures are eleven, twenty-six, and six, respectively. Central levels of the area pellucida including the germ wall gave for the ectoderm fourteen dorsoventral figures, thirty-eight anteroposterior, and six lateral ones; for the entoderm, three, twenty, and seven spindles, respectively.

These figures indicate that the posterior germ wall is the region of most active cell proliferation of the blastoderm and that it is increasing in thickness and adding cells anteriorly into the entoderm. The ectoderm of the pellucid area is spreading about equally in all directions by means of divisions both perpendicular and parallel to the surface. Since the ectoderm remains one-layered, it may be assumed that the cell products of vertically oriented spindles work into the ectoderm by rearrangement. The entoderm is less active than the ectoderm and is advancing more anteriorly than in other directions.

It should be remarked that the majority of the mitotic figures were in the metaphase. Since mitosis under the action of inhibiting conditions tends to pause in the metaphase, this suggests that the figures found were those present at the time of laying and not new mitoses initiated at room temperature. The fall of temperature after laying probably caused the early mitoses present to slow down and halt at the metaphase.

This study of the cellular disposition and mitotic frequency in unincubated blastoderms suggests strongly that the entoderm is advancing forward toward the anterior end of the area pellucida by proliferation from behind, particularly from the posterior germ wall. There was no evidence of an invagination in the posterior germ wall nor is any blastoporal split present, but the observations are not inconsistent with Patterson's account of the formation of the entoderm in the pigeon by a turning under at the posterior margin of the blastoderm (Patterson, '09). To determine the mode of origin of the entoderm in the duck, stages prior to laying must be studied.

FROM THE FIRST TO THE TWELFTH HOUR OF INCUBATION

Neither Lillie nor Wetzel discussed the early incubation stages of the chick to any extent. Duval's ('89) figures of five- and seven-hour blastoderms appear to be erroneous, for the embryonic shield is lacking and the blastoderm is shown

separated by clefts from the surrounding yolk. Mitrophanow's ('99) account of the chick appears to be correct and is here briefly abstracted. The germinal disk increases from a diameter of 3 to one of 4.5 to 6 mm., the area pellucida from 2 to 2.3 to 3 mm., by the eighth to ninth hour of incubation, at which time the primitive streak begins to appear. The thicker central ectoderm continues to thicken, reaching a height of 30 to 35 μ before the appearance of the streak. The ectoderm separates from the entoderm, beginning in the center and extending to the periphery. Schauinsland ('03) has figured a sparrow embryo in a prestreak stage. The section shows the central thickening of the ectoderm and a complete entoderm in the area pellucida.

Mitrophanow ('02) has described early incubation stages of the duck: five and one-half, six and one-half, nine, ten, and eleven hours. At five and one-half hours, the embryonic shield was present, 32 μ in height at its center. By six and one-half hours this had reached a maximum of 43 μ and was composed of clear cylindrical cells which had lost their yolk inclusions; the entoderm formed a continuous layer separated centrally from the ectoderm. At ten hours, the separation of ectoderm and entoderm was complete throughout the whole pellucid area. The primitive streak appears at the eleventh hour. My observations are similar to those of Mitrophanow, but are based on more complete series and more abundant material.

In general, the early development of the duck proceeds more slowly than that of the chick. The ectodermal shield of the duck is thicker, the entoderm does not become a coherent layer until ten to twelve hours of incubation, and the primitive streak appears at about twelve hours as contrasted with eight hours in the chick.

1. One- to three-hour stages

Twenty-four whole mounts and sixteen sectioned blastoderms of these stages were studied. There is practically no change from the unincubated state. The diameter of blasto-

derm and pellucid area have not altered. There is a slight diminution in the thickness of the germ wall, which averages $75.2\ \mu$ posteriorly and $39.3\ \mu$ anteriorly. Ectoderm and entoderm cells of the various regions practically correspond in height and extent to those of unincubated blastoderms.

2. Four to six hours

Study of twenty-four whole mounts and twenty-one sectioned series shows an increase in the diameter of the area pellucida and in the width of the area opaca. The former averages 1.32 mm.; the latter, 0.57 mm. The thickness of the posterior germ wall is again diminished, averaging $55\ \mu$ as compared with $83.5\ \mu$ of unincubated blastoderms. The thickened ectoderm of the middle posterior region of the pellucid area (embryonic shield) is higher than before, averaging $27\ \mu$ as compared with $25\ \mu$ of previous stages. In whole mounts the posterior half of the area pellucida appears a little darker, probably because of the thickened ectoderm of the embryonic shield and forward extension of the entoderm. The condition of the entoderm and the germ wall is the same as in the stage next to be described.

3. Seven to nine hours

The blastoderm has continued to increase in diameter, the area pellucida averaging 1.37 mm. in diameter, while the area opaca is 0.59 mm. wide. Study of twenty-one whole mounts shows that the dark area mentioned above has become larger and more conspicuous. It now occupies the posterior half of the area pellucida, presenting a convex anterior border rather sharply defined from the lighter-staining anterior part of the pellucida area. This darker-staining area is called the embryonic shield by many of the older writers. The name seems to have originated with von Baer. The darker appearance of this area is probably caused by the greater height of ectodermal cells and the greater thickness of the entoderm in this region. Examination of twenty sectioned blastoderms reveals but little change in the ectoderm from preceding stages.

The entoderm completely underlies the area pellucida and is much more coherent than in earlier stages, although still presenting gaps between the cells. It is several-layered in the posterior third of the area pellucida and thins anteriorly to a single loose sheet. The impression is gained that entodermal cells have migrated forward from the posterior thickening to fill the gaps in the entoderm. The germ wall is similar to the unincubated stage, thicker posteriorly than elsewhere, more or less syncytial, and mingled with yolk spherules. Beyond the germ wall, the free ectoderm extends farther than before as a rather flat membrane overlying the yolk.

4. Ten to twelve hours

The diameter of the pellucid area has increased to 1.63 mm and the width of the area opaca to 0.78 mm. Twenty whole mounts and twenty-three sectioned series of this age were prepared. Figure 2 is a photograph of a blastoderm of this age. As compared with the unincubated blastoderm (fig. 1), the distinction between area pellucida and area opaca is now evident. In the area pellucida the noteworthy feature is the circular dark area in the posterior central part. This is the embryonic shield or ectodermal thickening. It seems to have been mistaken by some workers for the earliest appearance of the primitive streak.

Sections show the embryonic shield to be composed of a thickened ectoderm which in its central portions is increased in height (28.9 μ) over preceding stages. The cells are tallest behind the center of the area pellucida and in front of the posterior germ wall and diminish toward the periphery. The cells of the shield are tending to become pseudostratified. The extent of the shield has enlarged over previous stages. The thickness of the posterior germ wall has remained about the same since the four-to-six-hour stage, about 55 μ . The entoderm now consists of a practically continuous layer of spindle-shaped cells. It is several layers thick posteriorly, single-layered anteriorly. Whereas in previous stages the entoderm cells were spherical, yolk-laden, and not in contin-

uity, they are now more flattened, less yolk-filled, somewhat spindle-like, and constitute a continuous layer.

Figure 10 is a drawing of a median sagittal section of a blastoderm of ten hours of incubation. It illustrates the points described in the preceding paragraph: the thickened posterior germ wall, the thick pseudostratified ectoderm of the embryonic shield, and the continuous entoderm layer, thickened posteriorly beneath the embryonic shield.

5. Abundance and distribution of mitotic figures

Counts have been made of the mitotic figures in the embryonic shield and underlying entoderm to throw some light on cell movements during prestreak stages. Posterior, middle, and anterior levels of the shield were studied in nine blastoderms. The anterior level lies at about the center of the area pellucida; the posterior, just in front of the thickened germ wall. As before, one section of each level was studied in each blastoderm and the mitotic figures found summed for the corresponding levels of the nine blastoderms. In the ectoderm there were counted a total in the nine specimens of three dorsoventral, forty-four anteroposterior, and forty-one lateral spindles in the posterior level; three, fifty-three, and twenty-seven, respectively, in the middle; and none, thirty-seven, and nineteen, in the anterior level. The preponderance of anteroposterior spindles indicates that cells are moving forward in the embryonic shield and the large number of lateral spindles suggests a spreading of the blastoderm peripherally and an addition of cells toward the center. It may be concluded that cells are moving from the periphery toward the center of the embryonic shield and forward in the shield. In the entoderm the figures were: for posterior levels, four dorsoventral, twelve anteroposterior, and four lateral spindles; for the middle levels, none, fifteen, and four, respectively; and for the anterior levels, none, five, and six, respectively. It is seen that the entoderm is not very active, for the number of mitotic figures has decreased since the unincubated stage. The majority of its figures suggest a forward

migration of cells from the posterior thickened region to fill the gaps in the more anterior levels of the entoderm.

PRIMITIVE-STREAK STAGES

That the bird embryo takes origin from a linear darkening in the midline of the area pellucida was known to the earliest investigators of embryology. Since the time of von Baer, this darkening has been called the primitive streak, after his designation. Waldeyer ('69) called attention to the fact, which he states had been overlooked by previous investigators, although represented by His ('68) in figures, that at its first appearance the streak is shorter, broader, and less definite than later. This early streak he designated the broad (*breite*) primitive streak; the later narrower, more elongated, and sharply defined structure, the narrow (*schlank*) streak. Mitrophanow ('99) has given a number of drawings and photographs of broad and narrow streaks of the chick. Wetzel ('29) gives a series of drawings of whole mounts and sections of various stages in the growth of the streak of the chick. According to him, and also Mitrophanow, the streak appears at about the eighth hour of incubation as a somewhat conical projection extending from the thickest region of the posterior germ wall forward along the median line of the circular area pellucida. This short primitive streak, as Wetzel designates it, at first extends only one-fifth to one-fourth of the diameter of the still circular pellucid area, but soon reaches half the distance and then presents a thickened anterior end, the primitive knot or Hensen's node, and behind this the primitive groove. By about the twelfth hour of incubation, the pellucid area has begun to elongate posteriorly into the typical pyriform shape, and primitive knot and groove are more pronounced. By sixteen to eighteen hours, the definitive narrow elongated streak has developed along the midline of the pyriform area pellucida, which is 3 to 3.5 mm. long. At the anterior end of the streak the primitive knot forms a thick, sharply bounded swelling. Hensen's node bearing a central depression, the primitive pit, from which the primitive

groove, bounded by the primitive folds, extends posteriorly and terminates near the posterior margin of the area pellucida, usually by a forked end. Dimensions are 1 mm. or a little less for the short primitive streak to 2 to 2.5 mm. for the definitive streak.

Primitive streaks of other birds—albatross, sparrow, starling—are illustrated by Schauinsland ('03). These resemble that of the chick, starting out as a short broad thickening which gradually elongates and narrows. The streak of the sparrow differs from that of other birds in that the rear end of early stages lies well within the area pellucida and does not make contact with the posterior germ wall. All subsequent workers except Duval are agreed that Koller's ('82) idea of the origin of the streak from a sickle-shaped thickening in the posterior germ wall is incorrect. Mitrophanow ('02) illustrates the primitive streak of a tern (*Sterna hirundo*).

The only work upon the early primitive streak of the duck is that of Mitrophanow ('02). He found the primitive streak appearing at ten or eleven hours of incubation, first as a darkened area in front of the posterior germ wall and later extending back to the germ wall. Later stages showed more elongated streaks, scarcely, however, narrower.

The primitive streak, as known to early observers, is a median ectodermal thickening in the posterior half of the area pellucida. It involves the thickened ectoderm of the previously existing embryonic shield, but otherwise bears no relation to the shield (Waldeyer, Mitrophanow). Mitrophanow ('99, '02), after careful studies of the earliest recognizable stages of the streak in duck and chick, states positively that the ectodermal proliferation begins at or a little behind the center of the area pellucida and at first does not reach the thick posterior germ wall. This is at variance with Wetzel's account, which is probably the most accurate. According to him, the ectoderm, previously one-layered, becomes several-layered in the streak, showing numerous mitotic figures above, and projecting below irregularly toward the

entoderm. The streak is widest posteriorly, but the thickening is deeper anteriorly (primitive knot). Cells proliferate ventrally from the ectoderm and fill the space previously present between ectoderm and entoderm. Wetzel's figures indicate, although this is not clearly stated in the text, that the proliferating band of ectoderm extends the length of the short primitive streak to the germ wall. The streak early becomes divisible into three regions: the primitive knot or Hensen's node, a thick proliferating region at the anterior end of the streak, closely bound to the entoderm, and containing the deep primitive pit; the Ebene, or less thick, looser region beyond the node, loosely connected to the entoderm and with a shallow, flat, primitive groove; and the broad, flat rear end of the streak, again bound to the entoderm and with a deep groove. In reporting my observations on the primitive streak of the duck, I shall divide the material into four arbitrarily selected stages.

1. Short primitive streak (Wetzel)

In the description of prestreak stages it was emphasized that the ectoderm of the central and posterior part of the area pellucida is thickened to form the embryonic shield. The height of the ectodermal cells of this shield increases during incubation until by ten to twelve hours the tallest cells are nearly 30μ in height. They tend to be pseudostratified. They lie behind the middle of the area pellucida and in front of the thickened germ wall. The primitive streak of the duck begins as a thickening and proliferation of this tall ectoderm of the embryonic shield.

Fifteen blastoderms in the earliest visible or short primitive-streak stage were studied. They were examined and sketched whole, and twelve were sectioned. The average diameter of the blastoderms was 3.4 mm.; of the area pellucida, 1.67 mm.; the length of the short primitive streak averaged 0.32 mm., or about one-fifth the length of the area pellucida. The time of incubation varied from nine to fifteen hours.

The first appearance of the primitive streak in the duck may occur at about nine hours, but usually is evidenced from the twelfth to the fifteenth hours of incubation. These observations accord with those of Mitrophanow ('02). Figure 3 is a photograph of the earliest visible or short primitive streak of the duck. The streak appears as a short, vaguely outlined, hemi-elliptical darkening in the posterior central part of the area pellucida. In some cases, as in the figure, the streak projects forward from the posterior germ wall; in other cases it does not in the whole mount appear to be connected with the germ wall. The area pellucida is still quite circular and scarcely enlarged since the last stages considered; but the blastoderm as a whole has enlarged, due to an increase in the width of the area opaca.

Sections through these blastoderms show that the primitive streak consists of a thickened and proliferating ectoderm. The ectoderm is thickest at the anterior end of the streak (average of $40\ \mu$) and diminishes posteriorly to $28.4\ \mu$ just in front of the germ wall. The streak is, however, broader posteriorly; the measurements of its width are $280\ \mu$ at the posterior end and $189\ \mu$ at the anterior end. Proliferation occurs along the entire length of the streak to the germ wall.

Sections of these early streaks present the following picture (fig. 12). At the anterior end of the streak the ectoderm becomes rather abruptly several-layered in the center of the blastoderm and forms a mass projecting ventrally toward the entoderm. The ectoderm cells of this mass are no longer of columnar form as previously, but polygonal. In the dorsal part of the mass the cells are compact and in close contact with each other; ventrally they become looser and more rounded and touch the entoderm. There is as yet no lateral migration of the cells as mesoderm. Outside of the streak the ectoderm is thickened and pseudostratified from the anterior part of the area pellucida back around the anterior two-thirds of the streak and laterally to the margin of the area pellucida. This thickened ectoderm, which can sometimes be seen in whole mounts as a slightly dark area, corre-

sponds to the Scheibe of Wetzel. It is considered by him the prospective medullary plate.

In two cases out of eleven a very shallow primitive groove was seen in the anterior part of the streak. It is shown in figure 12.

The entoderm is a coherent layer chiefly one cell thick. The posterior thickening is less marked than previously, but here the entoderm is still three or four layers thick. Elsewhere it is more or less one-layered with additional cells here and there (fig. 12). Anterior to the streak (fig. 11), the entoderm is thinner and less yolk-laden. There was no evidence of a definite Entodermhof (Wetzel) in front of the streak, where in the chick patches several cells thick occur. The entoderm of the duck may exhibit such patches of additional entoderm cells anywhere throughout the area pellucida. The entoderm is thicker posteriorly as in earlier stages.

The germ wall is much the same as previously except that it has extended considerably peripherally so that the thin margin of overgrowth is much wider than before. It is two or three cells thick and terminates with a pointed margin.

Figure 11 is a section through the blastoderm anterior to the streak showing the thickened ectoderm of the Scheibe and the continuous entoderm with occasional extra cells. Figure 12 passes through the center of the streak and illustrates the proliferating median region, the very thick ectoderm of the Scheibe, and the shallow primitive groove.

2. Medium primitive streak

This name is applied to stages in which the streak is longer than the preceding, about twice as long, but in which the area pellucida still preserves its circular contour. Rabl presents one embryo of this stage (his fig. 3), incubated twenty-four hours; length of the streak, 0.64 mm. The length agrees with my embryos of this stage, but the time of incubation is shorter in my cases. Rabl records that sections of this embryo revealed a very shallow primitive groove in the anterior part of the streak and a head process; the embryo

is thus more advanced than mine of the same length. I examined twenty-one embryos, seven as whole mounts, fourteen as serial sections. The average diameter of the blastoderm was 3.8 mm.; of the area pellucida, 1.68 mm.; the width of the area opaca averaged 1.06 mm. The streak (fig. 4) appears as an elongated club-like darkening in the center of the posterior half of the area pellucida. Its length averaged 0.61 mm., and it extends a third to a half of the diameter of the area pellucida. Around the anterior part of the streak there may appear a darker area probably representing the Scheibe. These stages were obtained during thirteen to twenty-one hours of incubation.

Sections reveal a picture similar to the preceding stage except that the proliferative thickening of the ectoderm occupies a longer area. Figures 13 to 15 illustrate sections anterior to the streak, through the anterior end of the streak and through its posterior end. The anterior end of the streak is $182\ \mu$ wide and $43\ \mu$ thick; the posterior end, $261\ \mu$ wide and $27.7\ \mu$ thick. As in the earlier stage, the posterior end is wider, but the thickening is deeper anteriorly. The main part of the streak (fig. 14) consists of a several-layered ectoderm bulging ventrally. The lower layers are looser and extend to the entoderm and some of the cells have become free as mesoderm. Anterior to the front end and continuous with the streak occurs a group of mesoderm cells proliferated from the ventral side and anterior end of the streak (not illustrated in the figures). These are called the anterior free mesoderm by Wetzell and the preaxial mesoderm by Adelman ('22). The ectoderm anterior to the streak (fig. 13) and around two-thirds of the streak (fig. 14) is thickened as the Scheibe. It presents the appearance of a pseudostratified epithelium. It is thickest at the sides of the streak ($31\ \mu$). It extends nearly to the margin of the area pellucida, where the ectoderm becomes a thinner ordinary epithelium ($18.2\ \mu$) and extends out over the area opaca as a flat membrane.

A shallow primitive groove was seen in three of the fourteen specimens in the anterior part of the streak. The con-

dition of the entoderm and germ wall is practically unchanged. The figures (13 to 15) illustrate their appearance.

3. Mitotic figures in early streaks

The distribution and orientation of mitotic figures in these two early stages of the streak were studied to ascertain possible cell movements. In serial sections of thirteen embryos examined under oil immersion mitotic figures were found in all levels of the streak and in regions immediately lateral to the streak. There were 115 spindles in anterior levels, seventy-one in middle levels, and sixty-eight posteriorly. This suggests that the anterior end of the streak is the most active proliferating region and that this activity decreases posteriorly (compare Hyman, '27). Further analysis of the spindles shows that there are sixty-seven anteroposterior spindles, thirty-two lateral spindles, and one dorsoventral spindle in a section through the anterior level of the streak. The majority (twenty-five) of the lateral spindles and also most (forty-nine) of the anteroposterior spindles are in the sides rather than in the main part of the streak. These data indicate a proliferation forward from the anterior part of the streak and from the sides toward the middle as well as forward. In the middle level of the streaks were counted a total of forty anteroposterior spindles, twenty-seven lateral, and six dorsoventral. Again the majority of the first two sorts lay in the sides of the streak. Posterior levels gave counts of forty-three anteroposterior spindles, sixteen lateral ones, and nine dorsoventral ones. Here the majority of the anteroposterior spindles were in the area pellucida, indicating forward movements here. Altogether the data suggest anterior movements in the streak and to the sides of the streak and lateromedial movements from the sides toward the middle of the streak. These findings are in accord with the conclusions of Wetzel and Gräper which will be discussed later.

4. *Elongated primitive streak*

This name is applied to rather elongated streak stages in which the area pellucida has begun to elongate posteriorly and assume the familiar pear shape. It somewhat corresponds to the broad primitive streak of Waldeyer ('69, fig. 9). Wetzel presents a good figure (fig. 14) of the chick of this same stage, and Rabl ('23) shows three duck embryos of corresponding appearance (figs. 4, 5, 6). These have pyriform pellucida areas with elongated streaks, averaging 0.9 mm. in length, which project from the posterior germ wall forward along the midline of the area pellucida. The streaks are surrounded by a faint circular area which Rabl regards as indicating the limits of the mesoderm. In whole mounts no primitive groove appeared, but sections showed that one was present in the three specimens of this age which Rabl studied.

My material of this stage comprises eighteen sectioned embryos and eleven whole mounts. They were obtained between thirteen and twenty-one hours of incubation. In a few cases this stage was seen as late as twenty-three to twenty-five hours. The average diameter of the blastoderm was 4.2 mm. The average length of the pear-shaped area pellucida was 1.8 mm. The streaks found in such pellucid areas varied in length from 0.45 to 1.03 mm., with an average of 0.9. Two-thirds of them were more than 0.7 mm. long.

In whole mounts (fig. 5) the area pellucida is rounded in front and narrowed posteriorly, presenting a pyriform or conical shape. The streak extends from the posterior bay of the area pellucida forward to about its center as a darker-staining bar. Its posterior end is wider and thinner than the anterior end. The anterior end, although darkly stained and rather definitely outlined, can scarcely be designated as Hensen's node at this stage. No primitive groove is apparent in whole mounts, although sections reveal its presence. The posterior part of the streak is more vague and less deeply stained than its anterior portion. The Scheibe is faintly evident as a darker area around and in front of the anterior part of the streak.

A series of sections through a primitive streak of this age is presented in figures 16 to 19. Figure 16 is taken just in front of the anterior end of the streak. It shows the thickened ectoderm of the Scheibe, extending to the margins of the area pellucida. This ectoderm has the appearance of a pseudostratified epithelium; it is composed of elongated cells with nuclei arranged in two levels. Below the central part of the Scheibe is seen the loose mass of the preaxial mesoderm. Figure 17 passes through the anterior end of the streak or Hensen's node, not as yet very sharply defined. The streak is here a narrow, well-delimited band of thickened ectoderm eight or nine cells thick, bulging ventrally to touch the entoderm. Its thickness here, including the attached mesoderm, varies from 24 to 56 μ , with an average of 49 μ ; its width averages 173 μ . The mesoderm forms the thick lower part of the streak; a few free mesoderm cells are beginning to wander laterally from the central mass. The Scheibe reaches the margin of the area pellucida as before. A section of the rear end of the streak is shown in figure 18; its thickness here is decreased to 20 to 32 μ , with an average of 23 μ ; its width averaged 300 μ . The streak is thus narrow, but deep anteriorly and wide and shallow posteriorly. The posterior thinning of the streak may be related to the backward extension of the margin of the area pellucida which carries the streak with it. At this level (fig. 13), the Scheibe no longer is present, the ectoderm outside the streak being an ordinary epithelium. Below the ectoderm are a few mesoderm cells. The entoderm throughout these sections is a thin coherent layer of flattened, spindle-shaped cells, with here and there extra cells above the regular layer. There is no definite Entodermhof, that is, region of thickened entoderm, anterior to the streak, as noted by Wetzell for the chick. At the posterior end of the streak (fig. 18), the entoderm is still thick as in early stages, consisting of several layers of yolk-laden cells. This passes directly into the thickened posterior germ wall, shown in figure 19, through the extreme posterior end of the streak, which is thus seen to overlie the germ wall.

The germ wall is about as previously, thick posteriorly, thinner elsewhere. The mesoderm has begun to migrate laterally from the primitive streak. In most of the cases (ten out of eighteen) there are a few scattered cells beginning to move laterally from the streak all along the streak. In two cases, the mesoderm has reached the area opaca in posterior levels of the streak. In the other six cases there is scarcely any migration of mesoderm.

The primitive groove cannot be seen in whole mounts, but the sections reveal its presence in the majority of cases as a very broad shallow depression, slightly deeper anteriorly. Of the eighteen sectioned embryos, ten showed a shallow groove, three gave some slight evidence of a groove, and in five the groove had not yet formed.

The mitotic figures were studied in thirteen streaks of this age and the total count was: 102 spindles in sections of anterior levels, ninety-eight in sections of the middle regions, and sixty-five in posterior sections. Again, the greatest proliferative activity occurs in anterior levels. When analyzed as to orientation of spindles, the data yield forty-six anteroposterior spindles, forty-six lateral spindles, and six dorsoventral spindles in anterior levels. Again the great majority of the anteroposterior and lateral spindles lay in the sides of the streak rather than in its center. This indicates not only general forward growth, but proliferation of cells from the sides into the axis of the streak. In middle levels, there were thirty-seven anteroposterior spindles, fifty-four lateral, and six dorsoventral. The preponderance of lateral spindles suggests marked movement from the sides into the center in middle levels of the streak. Posteriorly the mitotic activity is lessened, with twenty-nine anteroposterior spindles, twenty-nine lateral ones, and five dorsoventral. All levels show postero-anterior and lateromedial movements of cells.

5. Definitive primitive streak

This stage of the narrow, fully developed streak has been made familiar in the textbooks of embryology. Duval figures

it for the chick, Schauinsland for the sparrow and albatross, and Rabl for the duck. According to Wetzel, this stage is reached in the chick in sixteen to twenty hours of incubation, while Duval gives thirteen to sixteen hours. In the duck, Rabl presents similar stages obtained at twenty-nine to thirty hours of incubation. Schauinsland does not give the times of incubation. The primitive streak is similar in these various birds, consisting of a narrow, elongated rod-like body lying along the midline of the pellucid area. Its anterior end is thickened to form Hensen's node, and behind this the streak consists of the primitive groove bounded on each side by the primitive folds. The posterior end may be forked and, according to Wetzel, usually is so in the chick. In the chick, according to Wetzel, the definitive streak is about 2.5 mm. long, while the length of the area pellucida at this time is 3 to 3.5 mm. Rabl's average figures for his two duck embryos of this stage are 1.6 mm. for the pellucid area and 1.3 mm. for the streak. These figures agree very well with mine, but only one of Rabl's figures (fig. 6) presents a typical appearance with a fully developed groove. As in the other stages of development, the duck embryo is smaller than the chick.

In the duck, according to my results, the definitive streak is attained at eighteen to twenty-five hours of incubation. In twenty-two whole mounts the average diameter of the blastoderm was 5.70 mm. The area pellucida is decidedly pyriform, broad in front, narrow behind, with an average length of the area pellucida ranging from 1.4 to 2.7 mm.

The streak (fig. 6) appears as an elongated darkly stained rod extending from the posterior part of the area pellucida along the midline of the area. It occupies about three-fifths of the length of the area pellucida and averaged 1.2 mm. in length, with variations from 0.8 to 1.5. Its anterior end is thickened and deeply stained, forming the primitive knot or Hensen's node. Behind this is seen a clear streak, the primitive groove, bounded laterally by the primitive folds. The primitive pit is not evident nor is Hensen's node as well developed as in the chick. The posterior part of the streak

is often faint and often cannot be traced in whole mounts to the rear margin of the pellucid area. In some streaks whose length puts them into this stage, the primitive groove is not visible in whole mounts. Of the twenty-two specimens, the groove was deep in nine, shallow in six, and lacking in seven. Two showed no Hensen's node, either. The Scheibe is detectable in some specimens as a slightly darker area around the anterior third of the streak. The posterior end in the duck is never forked as seems to be commonly the case in the chick; but it is frequently curved or bent to one side. Of twenty-five embryos, the rear end of the streak was curved to the right in thirteen cases, to the left in six, and was straight in six.

Sections of eighteen embryos are available; figures 20 to 24 give various levels of a typical definitive streak with definite node and groove. Sections through the anterior part of the area pellucida show complete ectoderm and entoderm layers separated from each other. There is no mesoderm. The ectoderm here consists of a moderately tall epithelium, one-layered, slightly thickened toward the center and diminishing laterally to the edge of the area pellucida, where it becomes a thin membrane over the yolk. The entoderm is a continuous, somewhat flattened epithelial layer, with additional cells here and there. At the margins of the area pellucida, the entoderm becomes continuous with the germ wall. A little posteriorly, in sections shortly anterior to Hensen's node (fig. 20), the central ectoderm is seen to be much thickened and presents the pseudostratified appearance previously described, constituting the Scheibe, which extends nearly to the margins of the area pellucida. Below the central part of the Scheibe is seen the loose mesenchymal mass of the pre-axial or free mesoderm which is closely in contact with the entoderm and has begun to spread laterally (fig. 20). From this region there is a gradual transition to Hensen's node (fig. 21), characterized by the very thick pseudostratified ectoderm from the ventral surface of which cells are proliferating into a thick mass of mesoderm, consisting of loose

stellate cells extending to the entoderm. The pseudostratified ectoderm of the Scheibe here extends nearly to the margins of the area pellucida. The mesoderm is migrating laterally. At the posterior level of the node (fig. 22), the center of the streak shows a very shallow primitive groove, of the shape of a wide V. The central proliferating ectoderm and the mesoderm mass are slightly less thick here than at the anterior level of the node; but the mesoderm shows more extensive lateral migration, and has nearly reached the germ wall. It seems to migrate along the upper surface of the entoderm, which consists of a thin flattened membrane joining the germ wall laterally. Proceeding posteriorly (fig. 23), we find the groove more evident, the ectoderm of the streak less thick, and the mesodermal proliferation considerably diminished. The central ectoderm is still thickened and pseudostratified as the Scheibe, which, however, extends less than halfway to the margin of the area pellucida. The mesoderm forms a thin loose strip between ectoderm and entoderm, but is spread laterally throughout the whole area pellucida and has even penetrated into the area opaca. The entoderm is a thin membrane as before. At the extreme posterior end of the streak (fig. 24), the ectoderm is thin and has ceased proliferating mesoderm; there is no Scheibe. The mesoderm is a thin but dense layer and extends out over the germ wall. The entoderm is thicker and composed of two or three cell layers. There are irregular depressions in the ectoderm. The primitive groove continues to the posterior end of the streak.

The primitive streak of the duck can be divided into the same three regions emphasized by Wetzel for the chick: the anterior end with Hensen's node and the primitive pit; the flattened middle region, or Ebene, where the groove is shallow, and the posterior end, where the groove deepens again.

The germ wall has now for the first time become definitely organized into regions. At the margins of the area pellucida, it is protoplasmic, the protoplasm forming a network with large meshes, and with little yolk content. This may be called the germ wall proper, although it does not seem to be

cellular as stated by Lillie for the chick. It is continuous with the entoderm of the area pellucida. Lateral to this rather short protoplasmic region is a long zone of junction, composed also of a protoplasmic reticulum, but loaded with yolk, especially in its ventral part. It is overlaid by the very thin, membrane-like ectoderm. Outside of this is the margin of overgrowth composed of the membranous ectoderm above with a little yolky protoplasm beneath. This extends far out beyond the area pellucida. At the posterior end of the embryo the entoderm is continuous with the reticular protoplasm of the germ wall proper.

Measurements were made of various parts of the streak. The term streak here includes the ectoderm and the underlying attached mesoderm. Mesoderm cells which have become free and lie near the entoderm are not included. Through the anterior end of the streak the thickness varied from 40 to 72 μ , with an average of 56 μ . The width ranged from 80 to 200 μ ; average, 136 μ . Through the posterior part of the streak, the thickness varied from 10 to 32 μ ; average, 23 μ ; the width from 64 to 240 μ ; average, 154 μ . The streak is thus thicker anteriorly, but wider posteriorly. The ectoderm of the middle part of the Scheibe is 30 to 40 μ in thickness.

The distance of the anterior end of the streak from the anterior margin of the area pellucida diminishes during the development of the streak from an average of 1.35 μ in short streaks to 0.77 μ in the definitive stage. This indicates a forward progression of the streak, which is more marked than shown by the measurements, because meantime the area pellucida is expanding and thus moving away from the anterior end of the streak.

A study of the mitotic figures in thirteen definitive streaks, counted in the same manner as before, gave a total of 103 spindles at anterior levels, sixty-four in middle levels, and seventy-one near the posterior end. Again it is seen that the anterior end of the streak is the seat of the greatest proliferative activity. In the anterior end there were a total of forty-

four anteroposterior and forty-five lateral spindles. Through the middle the figures were twenty-four and forty-four, respectively, and posteriorly, twenty-nine and twenty-eight. This makes a total of 117 lateral spindles and ninety-seven anteroposterior spindles. As in previous stages, the majority of the spindles in anterior and middle levels lie in the sides rather than in the center of the streak. The results indicate postero-anterior and lateromedial migrations of cells.

HEAD-PROCESS STAGES

Head-process stages, preferably designated as primordial notochord stages, since the head process is the anlage of the notochord, not of the head, have been figured for many birds: for the chick by Duval and Wetzel, for the albatross, sparrow, and gannet by Schauinsland, and for the duck by Mitrophanow and Rabl. A total of thirty-two embryos were studied, fourteen as whole mounts, eighteen as sections. The area pellucida is much elongated, with an average length of 2.52 mm. The primitive streak, exclusive of the head process, averaged 1.32 mm., and has thus increased in length over definitive streak stages. The distance between the anterior margin of the area pellucida and Hensen's node has likewise lengthened to 1.2 mm., as compared with 0.77 mm. previously. The area pellucida has thus expanded, making it difficult to decide whether the streak has elongated in the anterior or posterior direction or both. It seems best to divide the head-process stage into two phases, early and late.

1. Early head process

According to Duval's atlas, this is reached in the chick by the nineteenth hour, with an area pellucida about 3.6 mm. long, primitive streak 1.7 mm., and head process 0.5 mm. These measurements were obtained from Duval's figure. In Rabl's four duck embryos of this time the area pellucida averaged 2.19 mm., the primitive streak 1.49 mm., and the head process 0.43 mm. in length. According to my findings, the head process first appears in the duck at about the

twenty-fourth hour. In thirty-two embryos the head process varied in length from 0.12 to 0.4 mm.; average, 0.20. Thus the duck requires longer to reach this stage than the chick and presents smaller measurements.

A duck embryo in early head-process stage is shown in figure 7. The area pellucida is elongated and pyriform, the primitive streak is in the definitive stage consisting of two parallel primitive folds, with the well-marked primitive groove between them. From Hensen's node a darkly stained short rod, the so-called head process, or primordium of the notochord, extends forward. In front of the head process is a transparent area devoid of mesoderm, and behind this a somewhat darkened area surrounding the head process and anterior half or two-thirds of the streak represents the Scheibe. The rear end of the streak is rather irregular and terminates in the germ wall.

Transverse and sagittal (fig. 25) sections of this stage show that the blastoderm anterior to the head process consists of a thin ectoderm and entoderm without mesoderm. Behind this the ectoderm over the head process thickens greatly into a pseudostratified epithelium with nuclei arranged in two or three layers. This is the primordium of the medullary plate, the former Scheibe. Below the anterior part of the medullary plate appears the scattered mesoderm of the prechordal plate (Adelmann, '22), thinner anteriorly, thicker posteriorly, and thinning again just in front of the head process. It extends some distance laterally in close contact with the entoderm. Behind the prechordal plate and continuous with it is the thicker, more compact mass of the chorda mesoderm (head process), eight to ten cells thick in front of the node, touching the medullary plate above, the entoderm below, passing posteriorly into the under surface of Hensen's node, and extending laterally as free mesoderm. The ectoderm of Hensen's node is a thick mass, which shows a slight depression tending to divide it into larger right than left portions; below there are still some indications of mesodermal proliferation. Behind the node the primitive pit forms a sharp

V-shaped depression in the very thick ectoderm of the Scheibe. Behind the pit, the streak consists of thick ectoderm two or three cells thick, which thins toward the posterior germ wall. Lateral to the anterior half or more of the streak the thickened ectoderm of the Scheibe extends to the margins of the area pellucida; but lateral to the posterior third of the streak, the ectoderm is scarcely thickened. Through middle levels of the streak the primitive groove is shallow and of thinner ectoderm; through posterior levels, its presence is barely indicated, but at the rear end it becomes deeper again and wider. The mesoderm is loose throughout the streak region, ten cells thick at the pit, thinning to three or four cells in thickness posteriorly. It extends laterally as thin loose sheets to the margins of the area pellucida at the pit level, in contact with the ectoderm out over the area opaca at more posterior levels. At the level of the posterior end of the streak, the mesoderm in the area opaca is greatly thickened into clumps preparatory to the formation of blood islands. The entoderm everywhere is a thin flat membrane, closely bound to the mesoderm of the head process and node, less in contact with it at middle levels of the streak and widely separated from the mesoderm at the rear end of the streak.

2. Late head process

The fully developed head-process stage is shown for the chick by Wetzel, for several birds by Schauinsland, and for the duck by Rabl. According to Duval's atlas, this stage is reached in twenty hours in the chick. Wetzel does not state the time definitely, but remarks that it occurs near the end of the first day. Rabl ('23) found it in the duck at twenty-nine to thirty hours; the average measurements of his three embryos are: length of the area pellucida, 2.65 mm.; length of the primitive streak, 1.7 mm.; length of the head process, 0.7 mm. I found that the duck embryo exhibits the maximum development of the head process at twenty-eight to thirty-one hours. Its length (determined from sections, since the structure is not clear in whole mounts) varied in twenty-one cases from 0.50 to 1.40 mm., with an average of 0.98.

The duck embryo at this time as seen in whole mounts (fig. 8) agrees very well with the drawings and descriptions of Wetzel (his fig. 32) for the chick and Rabl (his fig. 14) for the duck. The area pellucida is markedly pyriform. Behind the anterior transparent non-mesodermal area is the ridge marking the head fold with a darkly stained central part which represents the beginning of the brain. From this a paler band where the medullary folds arise extends back to the node. This whole nervous primordium is somewhat anchor-shaped. In it in late head-process stages the double ridge of the medullary folds, the right ridge always further developed and higher than the left, appears. The head process which lies beneath the medullary folds is scarcely visible in whole mounts except near the node. The node is a lens-shaped dark thickening cut in two longitudinally by a deep cleft, which passes through it asymmetrically so that the right part of the node is always larger than the left. Scheibe and streak are much as previously.

In studying the median sagittal section, the chief difference noted from the early head-process stage is that at the anterior end of the area pellucida, in the mesoderm-free region, the ectoderm and entoderm show a more marked elevation in what may now be termed the head of the embryo. The head fold or subcephalic pocket is indicated. In transverse sections of the head (fig. 26), the thickened elevated ectoderm of the future brain is underlaid by a thin entoderm on which rests in the median line the loose, laterally spread prechordal mesoderm. Through the level of the head process, the thickened medullary plate shows a central medullary groove, and right and left medullary folds, the right one higher and farther developed than the left. The head process is a compact mass below the medullary groove, to which it is strongly adherent, showing that it is notochord. Mesoderm extends lateral from the head process well out over the area opaca. The medullary groove becomes shallow over the posterior part of the head process and at the node passes into the primitive pit. The pit is a very deep cleft, almost touching

the entoderm below, slanted in such a manner that the right ectodermal fold of the node is higher and thicker than the left. Beneath the pit the mesoderm is scanty, but laterally it forms a thick layer extending out on the yolk and there thickening into blood islands. Posterior levels of late head-process stages are much like the earlier stage. The principal advance has been in anterior regions.

A neurenteric canal was noted by Balfour ('85, p. 163) in a duck embryo of twenty-six somites. I have not found any neurenteric canal in the stages studied, although the primitive pit in the late head-process stage is very deep, much deeper than that of chick embryos of corresponding degree of development. This fact at least suggests the possibility of the occurrence of a neurenteric canal in later stages.

The head-process stage is characterized in general by a decline of mesodermal proliferation from the streak and an increased activity of the ectoderm in forming the nervous system. The head process seems to arise as a forward migration of cells from the node. The greater activity of the right side of medullary plate and node is noteworthy.

HEAD-FOLD AND EARLY SOMITE STAGES

These stages are so well known in birds that only a few points will be mentioned. Rabl figured and described several duck embryos of these ages, which are in agreement with my observations. I have twenty-five whole mounts and twenty sectioned embryos belonging here. The head fold occurs in the duck around twenty-nine to thirty-three hours of incubation according to my and Rabl's findings, while in the chick as determined from Duval's plates, the time is about twenty-one hours. We may again consider the embryos in two stages.

1. Early head fold

This follows immediately upon the full development of the head process and differs from that stage chiefly by the formation of the head fold at the anterior end of the embryo. The head fold is caused rather by forward growth of the medul-

lary plate than by invagination of the proamnion—an explanation also given by Lillie for the chick. In whole mounts, the proamnion, the ridge of the head fold, its central darker brain thickening, and the medullary folds behind this are evident. In seventeen whole mounts of presomite head-fold stages, the right medullary fold was higher than the left in seven cases, while the two folds were approximately equal in the other ten. The medullary folds fade away toward the node. Node, pit, and streak are much the same as earlier. The posterior end of the streak is often irregularly sinuous and curved to one side. Of nineteen cases examined, nine were curved to the right, seven to the left, and three were straight. The Scheibe is still visible; blood islands appear around the posterior two-thirds of the area pellucida.

Sagittal sections show the head fold as a bay overhung by the thick anterior edge of the medullary plate, while the subcephalic pocket consists of the thin, widely separated ectoderm and entoderm of the proamnion. The anterior edge of the medullary plate is ten to sixteen cells thick and exhibits abundant mitoses indicating active proliferation. The prechordal mesoderm is proliferating anteriorly, as noted by Adelmann for the chick, and spreads laterally beneath the whole width of the medullary plate. In transverse sections are seen the well-developed medullary folds, equal or with the right one higher, the medullary groove with the round thick mass of notochord below, differentiated from the chorda mesoderm. The notochord joins the right and larger part of the node, while the medullary groove becomes continuous with the primitive pit. The streak still exhibits the three levels of Wetzel: anterior thick region with node and deep pit and groove; central Ebene with shallow groove, and posterior region where the groove is deeper again and the mesoderm thinner. The mesoderm is much the same as previously.

2. Early somite stage

The first somites appear in the chick at twenty-one hours, according to Duval; in the duck, as found by Rabl and me,

at about twenty-nine to thirty-five hours. I have three embryos showing the first intersomitic furrow, one incubated twenty-nine hours; two, thirty-two hours; six embryos with one somite, one of thirty hours, one of thirty-one hours, two of thirty-two and of thirty-four hours; nine with two somites, four of thirty-one hours, one each of thirty-two, thirty-four, and thirty-five hours, and two of thirty-three hours; and seven with three somites, three of thirty-three hours, two of thirty-one hours, and one each of thirty-five and thirty-six hours. The head fold deepens as the somites form and this relation is quite constant. The average diameter of the blastoderm was 9.31 mm.; length of the area pellucida, 3.21 mm. The primitive streak gradually decreases in length. In the head-process stage it averaged 1.32 mm. In the three embryos with the first intersomitic furrows it had decreased to an average of 1.3 mm. In the one-somite stage (six cases) it ranged from 1.2 to 1.4 mm., with an average of 1.28. At the two-somite stage (nine cases) the range was 1.0 to 1.4, with an average of 1.22 mm. For the seven three-somite specimens, the figures were 1.10 to 1.2 mm.; average, 1.13. The distance between Hensen's node and the anterior margin of the pellucid area is again increased, averaging 1.98 mm. This indicates either an elongation of the medullary-plate region or an expansion of the area pellucida.

As the anatomy of this stage presents nothing different from the well-known condition in the chick, a description of the sections is omitted.

RÉSUMÉ OF DEVELOPMENT

In the unincubated blastoderm of the duck, bilaterality has already been established as shown by a deeply stained crescentic area which is located at the future posterior end and is bisected by the later embryonic axis. This axis bears a relation which is approximately at right angles to the egg axis in slightly over 50 per cent of cases. This relation is preserved during incubation.

The unincubated blastoderm is composed of an outer, coherent, one-layered ectoderm and a lower incoherent entoderm, which is several layers thick posteriorly and thins anteriorly to a single layer. It consists mostly of separated cells. Encircling the area pellucida is the germ wall, much thicker at the future posterior end than elsewhere.

The diameter of the area pellucida and width of the area opaca remain unchanged during the first three hours of incubation, but thereafter increase at a gradually accelerating rate. The thick posterior germ wall decreases suddenly during the fourth to sixth hours to about three-fourths of its previous depth and remains in this state throughout the developmental period studied.

In the unincubated blastoderm the ectoderm of the posterior half of the area pellucida is slightly thickened as the precursor of the embryonic shield. This ectoderm increases in height during incubation and finally appears after several hours of incubation as the embryonic shield, which includes the ectoderm of the posterior half of the area pellucida. The primitive streak arises in the posterior part of the shield whose tall ectoderm becomes several-layered in the midline and begins to proliferate cells from its ventral surface. The streak is at first short and a little broad, but becomes longer and narrower until it attains the definitive stage. The primitive groove may appear at any stage of the streak, but it invariably is present only in the definitive stage; at first a shallow broad groove, it gradually deepens and narrows. Sections show that the primitive streak at all stages consists of a proliferating, thickened, several-layered ectoderm which occupies the whole length of the streak and reaches the germ wall posteriorly. The ectodermal thickening and proliferative activity are always greater at the anterior end of the streak, where at the definitive stage the thickening forms a definite mass, Hensen's node. The cells proliferated from the streak become the mesoderm. Study of mitotic figures shows that the greatest proliferative activity of the ectoderm is always at any stage in the anterior end of the streak (Hen-

sen's node) and diminishes posteriorly. The orientation of the spindles indicates postero-anterior and lateromedial migrations of cells. The elongation of the streak seems to be caused in part by more and more anterior ectoderm entering on proliferation and in part by migrations forward of cells that arose more posteriorly and migrations from the sides into the median line of cells that arose laterally.

The posterior part of the embryonic shield more or less disappears, but probably actually forms the rear part of the streak by migrating toward the midline. The anterior part of the original shield accompanies the anterior part of the streak as it grows forward and probably becomes the Scheibe or presumptive medullary plate. The Scheibe is a circular or oval area of very tall ectoderm which surrounds the anterior part of the streak. Later, as the time for the appearance of the medullary folds approaches, the Scheibe elongates and extends back about two-thirds of the length of the streak. The Scheibe is recognized by its very tall, pseudostratified appearance, with nuclei arranged in two or three strata.

Study of the number and orientation of mitotic figures in prestreak stages indicates that cells are moving from the rear part of the shield toward the midline and then moving forward in the midline. In the streak stages study of mitotic figures indicates that cells contribute to the middle regions of the streak by passing from the Scheibe toward the streak. The streak then elongates by the pushing apart of its ends through addition of cells to its central part.

The remainder of the Scheibe after these contributions to the streak is apparently all used up in the formation of the central nervous system. It seems to be drawn centrally as the medullary folds rise up and to be concentrated in them.

Hensen's node is a particularly thick and mitotically active part of the streak located at its anterior end. It is later cut asymmetrically by the deep primitive pit. Behind the pit the groove is shallow, then deepens again toward the rear end of the streak. The streak is thus divisible into three regions: anterior end, with Hensen's node and the deep pit:

middle region (Ebene of Wetzel), where the primitive groove is flat and shallow; and rear part, with a deeper groove. The primitive pit becomes continuous with the medullary groove and is always at the rear end of the latter.

The entire mesoderm arises from the lower side of the primitive streak along its whole length, apparently by actual proliferation of the ectoderm of the streak (see discussion). The mesoderm after it is formed begins to migrate laterally. This extension proceeds more rapidly at the posterior end of the streak and decreases anteriorly, so that the mesoderm always forms a pyriform area with the apex at the node. Migration laterally begins in the medium stages of the streak and progresses as the streak elongates. From the anterior and ventral side of the streak mesoderm extends forward in the midline as the free or preaxial mesoderm (prechordal plate). At the definitive streak stage the mesoderm at the node is thick, but does not extend very far laterally. Posteriorly it is thinner, but extends out beyond the margins of the area pellucida. In head-process stages the mesoderm extends at each side forward, so as to form horns enclosing the proamnion. The head process consists of mesoderm cells which migrate forward from the right side of Hensen's node and become the notochord.

The entoderm in the unincubated blastoderm is a loose layer of separate cells, several cells thick posteriorly, one layer thick throughout most of the area pellucida. It consists of separate rounded cells with many gaps between them, but underlies the whole area pellucida. Its posterior thickened part is continuous with the posterior thickening of the germ wall. Study of mitotic figures indicates that this posterior germ wall is the most active region of unincubated blastoderms and is adding cells into the entoderm. There was no evidence of the formation of the entoderm by an invagination, nor was any blastoporal split present; the observations indicate a formation of entoderm by forward proliferation from the posterior germ wall.

The entoderm becomes filled in to form a continuous coherent sheet during early incubation apparently by migration of cells from the thick posterior region of the entoderm. This process is complete just before the appearance of the primitive streak.

The entoderm is closely fused to the preaxial mesoderm, the head process, and Hensen's node, but is more or less separate from the mesoderm elsewhere.

There was no evidence of a definite Entodermhof or region of two-to-three-layered entoderm anterior to the primitive streak as described for the chick.

The entoderm continues everywhere into the germ wall, which is rather unorganized in early duck embryos, and cannot be differentiated into germ wall proper and zone of junction. This distinction appears first in the definitive streak stage, where the inner boundary of the germ wall becomes a protoplasmic reticulum, while its more peripheral portions remain yolk-laden. The germ wall throughout is thicker at the posterior end of the embryo than elsewhere and in early stages is here a region of mitotic activity.

At all of the stages studied the duck embryo is of smaller dimensions than chick embryos of the same degree of development and requires longer to attain the various stages than does the chick. The primitive streak in the duck begins at about the twelfth hour, the head process at the twenty-fourth hour, the first somites at twenty-nine to thirty-five hours.

DISCUSSION

1. Origin of the entoderm

Since in the unincubated blastoderm with which stage this study began the entoderm is already present, its origin was not definitely determined. The observations, however, are suggestive. The facts that the posterior germ wall is thickened and proliferating, that the entoderm is thick and many-layered in front of this, and thin anteriorly, and that the majority of the mitotic figures are oriented in an anteroposterior direction indicate that the entoderm is being formed by

the posterior germ wall and is pushed anteriorly by proliferation from behind. There was no evidence of a blastopore-like cleft in the posterior germ wall nor of an invagination process. The accepted theory of entoderm formation in birds by a gastrulation process at the posterior end of the blastoderm, in which the entoderm turns under and grows forward, rests almost entirely upon the observations of Patterson ('09) for the pigeon. My findings on the duck, while not consistent with Patterson's theory, do not lend any direct support to the existence of an actual invagination. They indicate a simple forward growth from a thickened posterior region.

2. Origin of the primitive streak

Numerous theories of the origin of the streak have been advanced, but practically all of the older ones are variations of the idea, which seems to have originated with Rauber ('76, p. 568, figs. 8, 9), that the streak is an elongated blastopore and is formed by the concrescence of the posterior margin of the blastoderm to either side of the middle line. In this category belongs the theory of Koller (pp. 186, 193) that the primitive streak arises by the forward growth of the central thickened part of a sickle-shaped ectodermal area (Sichel) found at the boundary of area pellucida and opaca at the posterior end of the blastoderm. Essentially similar is Duval's theory of the origin of the streak from the axial plate, a region at the rear end of the area pellucida where ectoderm is in contact with the underlying elements and in which the primitive streak arises by concrescence. The concrescence theory, probably based on erroneous observations, in part on abnormal embryos, has persisted to the present time as the chief theory of the formation of the streak. It was objected to by Mitrophanow ('02, p. 194), who claimed from a study of sections that in both chick and duck the ectodermal thickening which forms the streak arises near the center of the area pellucida and does not extend to the posterior germ wall. This does not agree with my findings for the duck nor Wetzel's for the chick. According to our observations, the primitive

streak appears morphologically as a slightly elongated thickening in the posterior part of the area pellucida and extends back to the posterior germ wall. The whole length of the streak is proliferating from the beginning. The streak is at first short and a little broad and extends about one-fifth the length of the area pellucida. The very broad hemispherical area depicted by Hoadley ('26), found by him in the chick at four hours' incubation, and considered by him to represent the earliest appearance of the primitive streak, is probably nothing but the embryonic shield. No other authors have figured such a streak in any birds, nor does the streak of the chick appear at such an early stage of incubation. The short streak consists of a many-layered ectoderm proliferating cells from its lower surface. The thickness of the ectoderm and its proliferative activity are greatest at the anterior end of the streak and decrease posteriorly, but both continue into the posterior germ wall.

Whether the streak actually arises in situ cannot be settled by purely morphological observations. The marking experiments of Wetzel on the chick indicate that in early blastoderms there is cell migration from the posterior lateral parts of the area pellucida toward the middle and along the midline from the posterior end forward. Gräper in his moving pictures of chick embryos of prestreak stages found a similar state of affairs. There was movement in the posterior part of the area pellucida from the lateral regions toward the middle and forward along the middle regions. Thus the streaming in the ectoderm has the form of a double whorl, backward along each side to the posterior end and forward along the middle. My data on the abundance and orientation of mitotic figures in prestreak duck embryos are in agreement with Gräper and Wetzel. The preponderance of anteroposterior and lateral spindles in posterior levels suggested cell movements from the sides toward the middle and from posterior regions forward along the midline. It thus appears that the cells of the short streak are not the same as those that earlier occupied this position, but have moved in from the sides.

Gräper states that the primitive streak is formed by these streaming movements, although it is not at all clear how he conceives this, unless he means that the cells heap up. The streak first appears in the center of the forward streams as a pit with sharp front and sides over which material streams down into the pit. It appears to me that neither Gräper nor Wetzel explains how the streak is formed by these movements.

After its first appearance the streak elongates and becomes somewhat narrower. We are here confronted with two questions. Does the streak elongate at its anterior or its posterior end or by its ends being shoved apart through elongation of the middle? And is the elongation the result of migration of the previously proliferating cells or do new levels of cells at anterior or posterior ends or both which were not previously proliferating begin to proliferate? The marking experiments of Wetzel have shown that marks placed in front of young streaks are moved forward and to the side, indicating a forward extension of the streak, in early stages. Marks at the rear show a backward movement at the time of the first elongation of the area pellucida. Thus early streaks are elongating at both ends. The elongation of the later streaks, however, is due chiefly to migration of cells from lateral regions into the middle part of the streak, pushing the two ends apart. Gräper states that at first the streak lengthens by the addition of material to its rear end through the streaming already described. Soon, however, the double whorl is replaced by lateromedial streaming. Material from the sides streams into the middle part of the streak, passes over the primitive folds down into the groove, and then turns and passes outward again as mesoderm. The results of Gräper and Wetzel therefore agree. My data on mitotic figures of early streaks indicate movements anteriorly in the streak and from the sides toward the middle; in definitive streak stages the greatest movements seemed to be from the sides into the middle.

Nevertheless, we do not get a clear picture of the growth of the streak. If the cells are continually moving, how do we

obtain a definite morphological picture? Is the ectoderm of the streak actually not proliferating at all, but only serving as a place where cells heap up and migrate down into the interior? Gräper seems to think this is the case. But my findings of abundant mitoses in the streak indicate actual cell proliferation, especially at the anterior end. It does not seem possible to answer the question whether hitherto inactive cells become involved in the streak activity and whether the original proliferating cells move into new situations. I am of the opinion that both processes occur. Morphological study shows merely that the band of apparently proliferating cells elongates.

From the work of Gräper and Wetzel and my own study it may be concluded that while it is true that cells are added to the streak from the sides, this does not occur in accordance with the concrescence theory. This theory of the formation of the streak is therefore incorrect, as far as the actual facts of present development are concerned. As to how the primitive-streak mode of development common to Amniotes may have evolved phylogenetically from the amphibian mode cannot be answered. In its physiological activity and rôle in development, the streak does seem to correspond to the dorsal lip of the amphibian blastopore (compare Wetzel, p. 280).

3. Formation of the node

Neither Gräper nor Wetzel presents any specific data on the formation of the node. Apparently this structure is merely a thicker and more actively proliferating portion of the streak, as shown by its appearance in section and the number of mitotic figures. It is not clear from Gräper's account of cell migration how this region happens to be thicker. One may suppose that in the forward migrations of cells they heap up in this region. Mitotic figures in my sections show clearly additions of cells to the median region by lateral mitoses. Sections of the node show more mitoses just at the sides of the node than in the node itself. The node may then represent a region of accumulation of cells moving in from behind and from the sides.

4. Origin of the head process

It seems evident from morphological study that the head process is simply a mass of mesoderm which migrates forward from the anterior and ventral surfaces of the node. This view is accepted in Lillie's text and is confirmed by Wetzel's marking experiments. It cannot be formed by a backward moving of the node, leaving the head process in front of it, because at this time the streak is at its maximum length and shows no evidence of any shortening. The backward progression of the node does not begin until after the head process has been fully formed.

5. Later stages

Direct observations together with the moving pictures of Gräper and the marking experiments of Wetzel leave no doubt that after the head-process stage, the node moves rapidly backward until the somites are completed. This is evidenced in my study by the progressive shortening of the primitive streak and lengthening of the medullary-plate region. The rear end of the nervous system is always at the primitive pit. As the node moves back it deposits the notochord in front of it. The notochord can always in sections be traced into the node, with which it remains continuous during its formation. It seems rather strange that up to the last few years this rather obvious process of backward retreat of the node should have escaped the attention of embryologists.

Since the results have been summarized in a résumé preceding the discussion, a formal summary is omitted.

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ABBREVIATIONS

<i>a</i> , anterior end of blastoderm	<i>p</i> , posterior end of blastoderm
<i>ec</i> , ectoderm	<i>pf</i> , primitive fold
<i>en</i> , entoderm	<i>pg</i> , primitive groove
<i>gw</i> , germ wall	<i>pm</i> , preaxial mesoderm
<i>hp</i> , head process	<i>pp</i> , prechordal plate
<i>m</i> , margin of overgrowth	<i>ps</i> , primitive streak
<i>mc</i> , mesoderm	<i>pt</i> , primitive pit
<i>mp</i> , medullary plate	<i>s</i> , Scheibe
<i>n</i> , Hensen's node	<i>sh</i> , embryonic shield

The drawings of plates 3 and 4 were made at an initial magnification of 100 diameters and reduced to 50 diameters in reproduction.

PLATE 1

EXPLANATION OF FIGURES

- 1 Photograph of an unincubated blastoderm (41-1), showing the posterior thickened germ wall. $\times 28$.
- 2 Photograph of a blastoderm in the shield stage (17-11). Age, eleven hours. $\times 28$.
- 3 Photograph of a blastoderm in the short primitive-streak stage (84-11). Age, fourteen hours. $\times 18$.
- 4 Photograph of a blastoderm in the medium primitive-streak stage (12-18). Age, sixteen hours. $\times 23$.

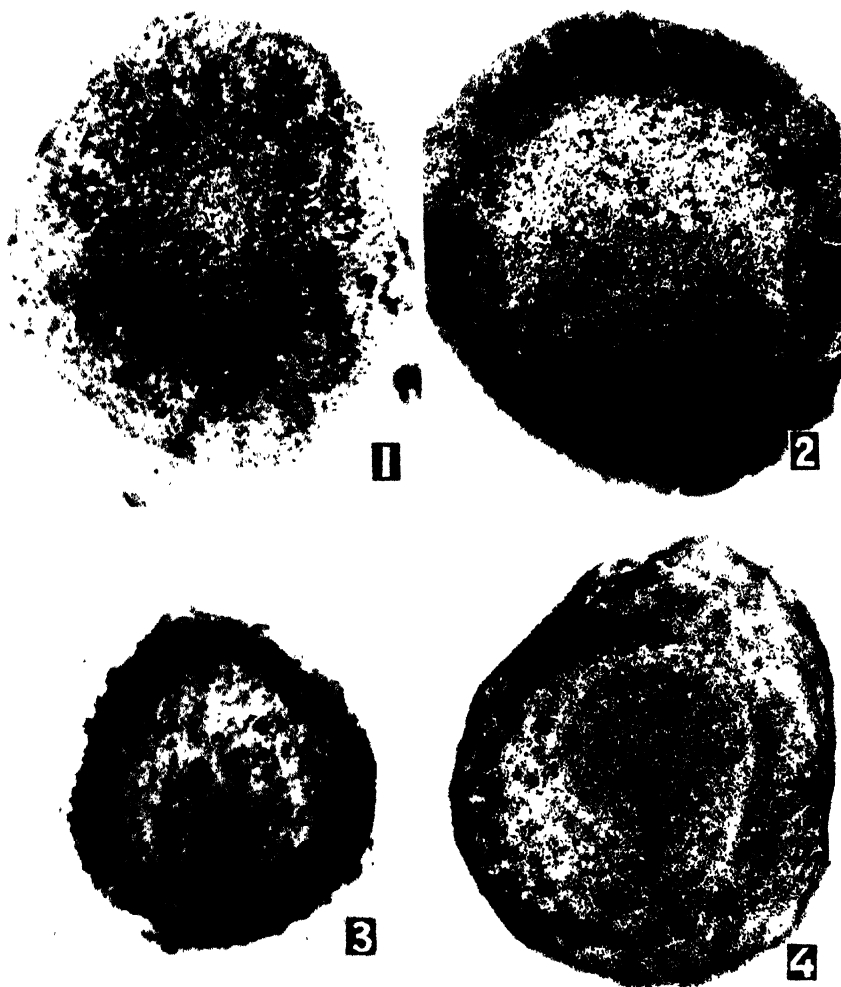


PLATE 2

EXPLANATION OF FIGURES

5 Photograph of a blastoderm in the elongated primitive streak stage (14-19). Age, nineteen hours. $\times 23$.

6 Photograph of a blastoderm in the definitive primitive-streak stage (10-25). Age, twenty-four hours. $\times 26$.

7 Photograph of a blastoderm in the head-process stage (16-27). Age, twenty eight hours. $\times 20$.

8 Photograph of a blastoderm (35-31) in medullary plate stage. Note anchor shaped medullary plate extending from right half of the node. Age, thirty one hours. $\times 20$.



PLATE 3

EXPLANATION OF FIGURES

9 Median sagittal section of an unincubated blastoderm (26-1). Note especially the thickened posterior germ wall (compare fig. 1).

10 Median sagittal section of a blastoderm in the shield stage (9-8), corresponding to stage shown in figure 2. Age, ten hours. Note embryonic shield and its relation to the thickened germ wall, the extent of the margin of overgrowth, and that anteriorly the entodermal cells are not yet formed into a coherent layer.

11 and 12 Transverse sections through a blastoderm in the short primitive-streak stage (4-13), corresponding to stage shown in figure 3. Age, twelve hours.

11 Through the area pellucida anterior to the streak.

12 Through the middle level of the streak.

13 to 15 Transverse sections through a blastoderm in the medium primitive-streak stage (3-31), corresponding to stage illustrated in figure 4. Age, seventeen hours.

13 Through a level of the area pellucida anterior to the streak.

14 Through a level of the anterior end of the streak. Note slight amount of mesoderm below streak.

15 Through the posterior end of the streak.

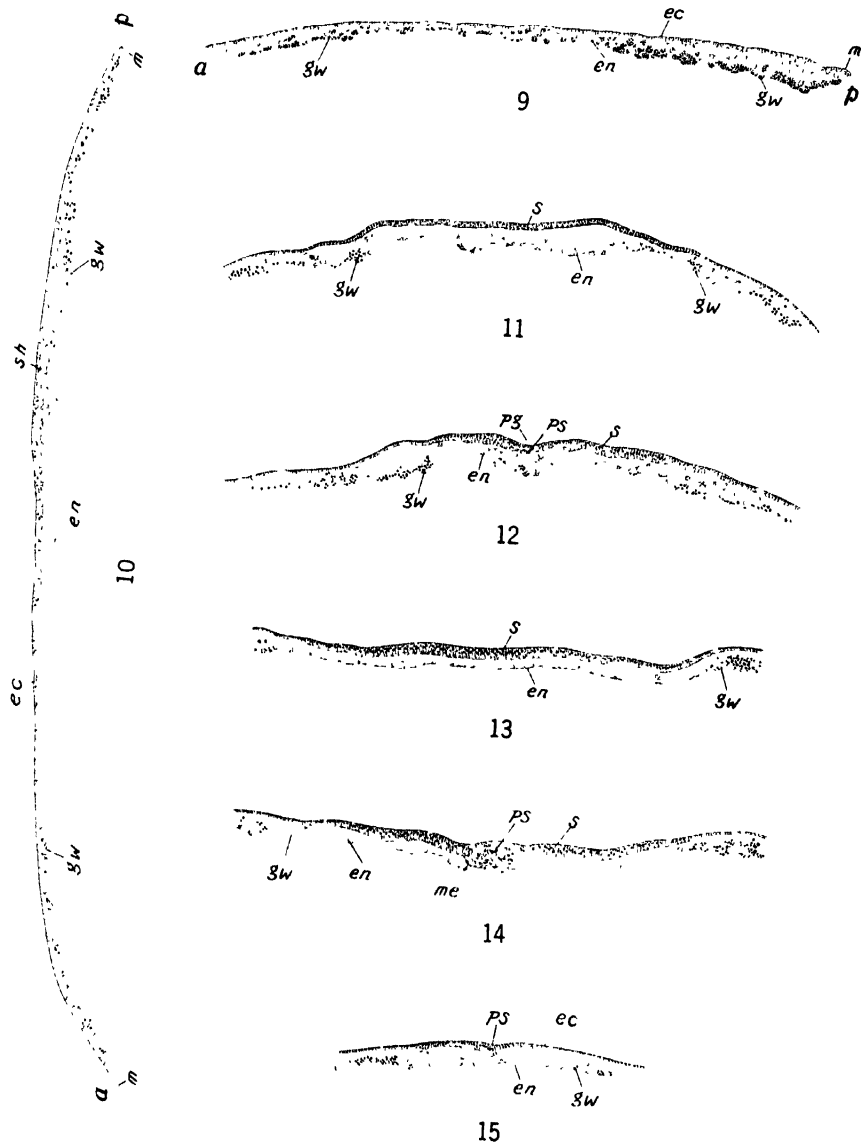


PLATE 4

EXPLANATION OF FIGURES

16 to 19 Transverse sections through a blastoderm in the elongated primitive-streak stage (36-13), corresponding to the stage shown in figure 5. Age, twenty hours.

16 Through the area pellucida anterior to the streak. Note Scheibe and preaxial mesoderm.

17 Through the anterior end of the streak.

18 Through the posterior end of the streak.

19 Through the extreme posterior end of the streak above the germ wall.

20 to 24 Transverse sections through a blastoderm in the definitive streak stage (10-21), corresponding to stage shown in figure 6. Age, twenty three hours.

20 Through level of the area pellucida anterior to the streak.

21 Through level of Hensen's node.

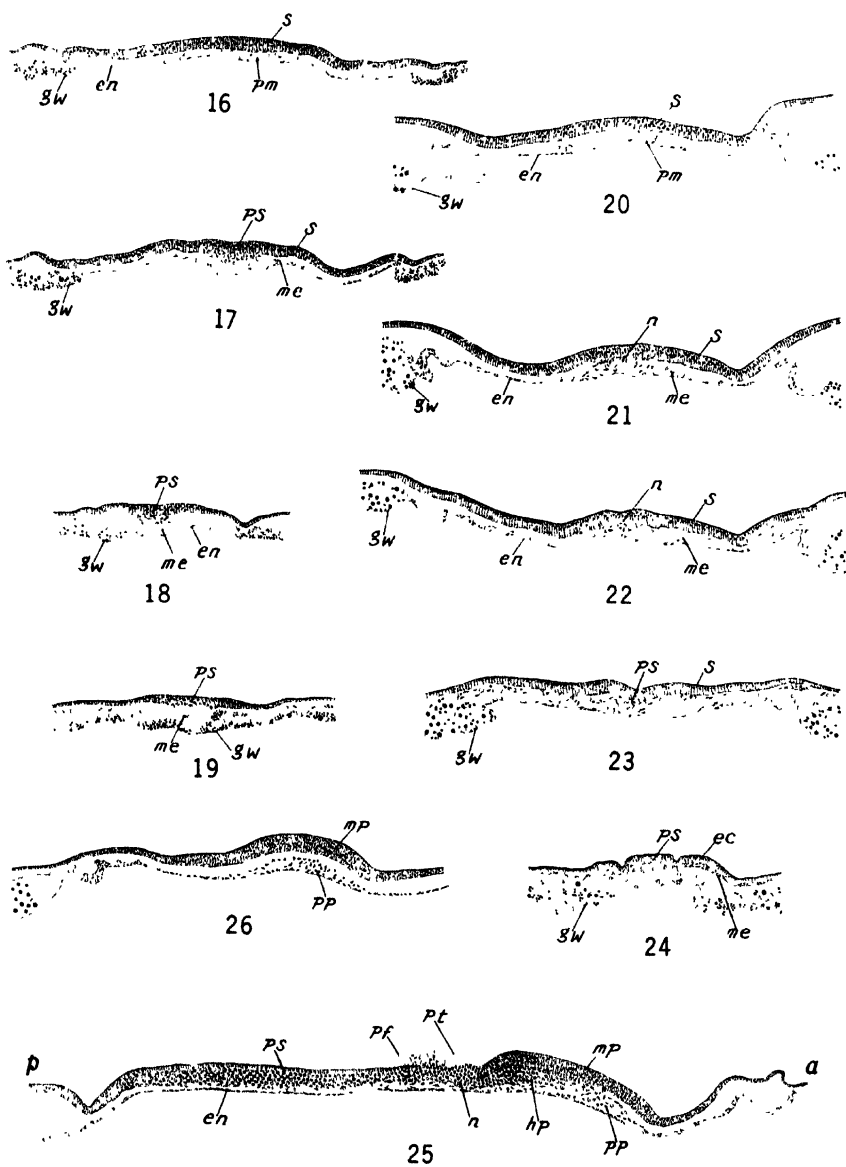
22 Through the posterior level of the node.

23 Through the middle level of the streak.

24 Through the posterior end of the streak.

25 Median sagittal section of a blastoderm in the head-process stage (4-23), corresponding to stage shown in figure 7. Age, twenty-seven hours.

26 Transverse section through the anterior end of the medullary plate of a blastoderm in the late head-process stage (15-32), corresponding to stage shown in figure 8. Note especially the thickened medullary plate and the prechordal plate. Age, twenty-nine hours.



A COMPARISON BETWEEN THE ULTRAVIOLET MICROSCOPY AND THE FEULGEN STAINING OF CERTAIN CELLS

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THREE PLATES (TWENTY-FOUR FIGURES)

AUTHORS' ABSTRACT

This paper describes ultraviolet (λ 2750 Å) photomicrographs of resting and dividing chicken macrophages and fibroblasts and of erythrocytes and lymphocytes. The structures found in these photographs are compared with the ones brought out in fixed material by Feulgen staining and found to be essentially similar in appearance. A preliminary series of ultraviolet pictures is also shown of a single fibroblast passing through several of its stages of division.

One of the most important advantages of ultraviolet microscopy resides in its ability to show in living material many of the structures ordinarily brought out in stained and fixed preparations. By doing this it can tell conclusively which of the details in the stained figures have reality within the living cell and which are meaningless coagulation artifacts produced by the reactions of fixing and coloring.

Chromosomes are far more opaque to the ultraviolet light used in micrography than are most other constituents of living material. Accordingly it has seemed important to compare ultraviolet photomicrographs of several kinds of cells with preparations stained specifically for chromatin. Some of these results are contained in this paper. In order to be sure that the cells photographed were healthy and not freshly killed or seriously damaged by radiation, series of ultraviolet exposures have been made of a single cell passing normally through the various stages of mitosis.

TECHNIQUE

The ultraviolet microscope has been used for previous experiments¹ in this laboratory. It consists of a Barnard instrument fitted with Zeiss objectives and eyepieces and carrying a modified Leica camera for photography upon motion-picture film. The light source was the 2750 Å line from a condensed cadmium spark, isolated by a simple prism spectroscopic arrangement. The desired field was found with the green line of a mercury arc and sharply focused for 2750 Å by applying an empirical correction. In order to make accurately timed short exposures, a suitable shutter has been interposed between the light source and the condensing lens. Because ultraviolet radiation is destructive to all living cells it is important that material being studied should receive as little as possible of this light. Brief exposures are also essential because under existing experimental conditions a limit to the detail seen in living matter is set not so much by the resolving power of the optical system as by movement within the cells themselves. For both these reasons the final photographs are enlargements from very fine-grained negatives of relatively low initial magnifications.

Four cell types, all from the chicken, were examined—fibroblasts, macrophages, lymphocytes, and erythrocytes. The first two were studied in pure culture, cultivated by the usual methods. The others were found in adult chicken blood. Examples of these cells also were photographed in fresh preparations of embryonic spleen. Most of the photographs of lymphocytes and red cells were obtained from suspensions in Tyrode solution that had been placed between a quartz cover-glass and slide and sealed with vaseline or paraffin. The pure strains and spleen tissue were grown within small containers formed by covering both faces of a drilled glass slide with quartz cover-glasses. The plasma clot in which these cells proliferate must be as thin as possible because of its strong ultraviolet absorption.

¹ R. W. G. Wyckoff and A. L. Ter Louw, *J. Exp. Med.*, vol. 54, p. 449 (1931).

The Feulgen reaction² was chosen as the means of staining chromatin for comparison with ultraviolet photographs. Samples of each cell type were fixed in alcohol and acetic acid, hydrolized in HCl, and stained with the monaldehyde derivative³ of sulphonated pararosanilin. Under this treatment formed chromatin takes on an intense violet coloration. Photographs of some of these stained preparations were made with green light from a mercury arc using a photomicrographic camera equipped with a Zeiss 3 mm.-N.A. 1.4 apochromat objective and an 8× Leitz periplan ocular.

Prints from typical photographs with ultraviolet light upon living and with green light upon stained material are reproduced in plates 1 to 3. In most instances these prints were made from duplicate negatives using process film. Additional details concerning individual photographs are to be found in the accompanying descriptions.

Sequences of ultraviolet photographs showing a cell passing through several stages of mitosis were made for both fibroblasts and macrophages. Part of such a series, sufficient to prove that cells are not killed by the amount of ultraviolet light necessary to photograph them, is shown on plate 3.

DISCUSSION

In resting fibroblasts the nucleolus alone appears to absorb ultraviolet light. Even so, its opacity is much less than that of the chromatin of dividing cells. The nucleus itself, though it can be seen by reason of the different texture of its protoplasm and the absence within it of the many droplets which fill a large part of the cytoplasm, has practically the same transparency as the rest of the cell. Within the nucleus, however, irregularly outlined specks of absorbing material are often seen. It is important to notice that similar bodies are the only elements which appear on Feulgen staining (fig. 5). The nucleolus does not give the Feulgen reaction but counterstains with the cytoplasmic dye light green (figs. 6 and 7).

² B. Lee, *Microtonist's Vade Mecum*, 9th edition (Philadelphia, 1928), p. 306.

³ E. Wermel, *Zeit. f. Zellforsch. u. mikroskop. Anat.*, Bd. 5, S. 400 (1927).

Fibroblasts are not readily killed by ultraviolet light. This is clearly shown by the apparently healthy condition of cells which have received for hundreds of seconds the concentrated illumination from the microscope condenser (fig. 3). With too much irradiation, however, they disintegrate by the rupture of their walls and the extrusion of increasingly large masses of protoplasm. A blister of this sort is shown in figure 4.

Macrophages are far more sensitive to ultraviolet radiation. The cell of figure 12 is already ruptured at several points; complete disintegration followed soon after this photograph was made. In resting macrophages, as in fibroblasts, the nucleus and its surrounding cytoplasm are about equally absorbing to ultraviolet light. Corresponding to this nuclear transparency is the absence of an intense Feulgen staining (fig. 14) except for a few isolated particles which also appear black in the ultraviolet photographs.

The ultraviolet opacity of the formed chromatin in dividing fibroblasts and macrophages is in striking contrast to these transparent nuclei. The rest of a cell does not seem to become more transparent as absorbing chromatin forms within it. This suggests that a chemical reaction productive of the strongly staining and absorbing chromatin occurs during the early stages of mitosis.

Unlike fibroblasts and macrophages whose nuclei are transparent except during division, the non-dividing erythrocytes and lymphocytes of the chicken have nuclei which at all times contain strongly absorbing bodies. This is true of cells both from early embryos and from adults. Feulgen staining brings out exactly the same structures in these cells (figs. 11 and 15). It will be of interest to determine whether all nucleated non-dividing cells have similar permanent chromatin networks.

The cytoplasm of fresh red cells is moderately absorbing for ultraviolet light. After some exposure to radiation, however, it becomes nearly transparent (fig. 9). This bleaching is scarcely to be explained as due to protoplasmic losses

through a ruptured cell wall since the cell outline remains unaltered.

We are indebted to Dr. A. Carrel for the interest he has taken in the carrying out of these experiments.

CONCLUSIONS

Ultraviolet ($\lambda = 2750 \text{ \AA}$) photomicrographs have been made of resting and dividing chicken macrophages and fibroblasts and of erythrocytes and lymphocytes. These pictures have been compared with similar fixed preparations stained by the Feulgen reaction. All the gross structures brought out by Feulgen staining have been found in living material by their intense ultraviolet absorption. Although this material is not suitable for comparisons of the finest detail, no significant differences were discovered between the living and the fixed preparations.

The nuclei of the non-dividing erythrocytes and lymphocytes of the chicken are at all times filled with more or less continuous filaments of chromatin. No absorbing matter can be seen in resting fibroblasts and macrophages but as mitosis begins, intensely absorbing chromosomes gradually make their appearance. The nucleolus is the only other cell organ which absorbs ultraviolet light. Its absorption is, however, so much less intense than that of formed chromatin that there is little chance of confusion. For these cell types, then, ultraviolet microscopy can be considered as the equivalent of a vital staining specific for formed chromatin.

Series of pictures of a single cell passing through its several stages of mitosis show that material photographed by ultraviolet light is capable of continued normal growth and multiplication. It is obvious that this ultraviolet 'motion picture' technique can provide definite and conclusive information concerning many details of cell division.

PLATE 1

EXPLANATION OF FIGURES

1 A fibroblast from embryonic chicken spleen fixed in a thin plasmatic clot and flooded with Tyrode solution. Incubated twenty-four hours and photographed (with ultraviolet light) at room temperature. Previous exposure: eight seconds. $\times 1200$.

2 A cell from a subculture of a nineteen-year-old strain of fibroblasts ('Old Strain') photographed (with ultraviolet light) at room temperature. Previous exposure: fifteen seconds. $\times 1200$.

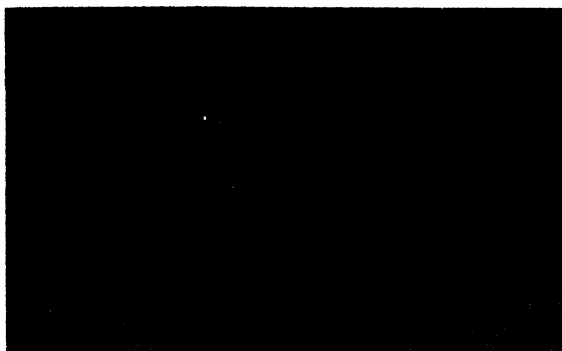
3 A fibroblast from embryonic chicken spleen photographed (with ultraviolet light) at room temperature. Previous exposure: 240 seconds. Even after this heavy irradiation the cell showed no sign of breaking up. $\times 1200$.

4 A fibroblast from embryonic chicken spleen photographed (with ultraviolet light) at room temperature. Previous exposure: 320 seconds. $\times 1200$. After this prolonged irradiation many neighboring cells showed blisters similar to the one in this photograph. The nucleolus, which at first was difficult to see with ordinary light, was easily visible at the end of the experiment.

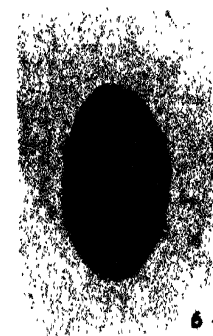
5 A cell from a subculture of 'Old Strain' fibroblasts fixed and stained with Feulgen reagent and counterstained with light green. The photograph was made on a Wratten 'M' plate with light from a mercury arc filtered through a Wratten no. 77 filter. Little more than the nucleus is shown. $\times 1200$.

6 A fibroblast near the one photographed in figure 5. The same technique has been used. In this figure the green-staining nucleoli can be faintly seen.

7 The same cell as the foregoing photographed with mercury light through a Wratten no. 77 filter and a gelatin film stained with eosin and picric acid to bring out more clearly the green-staining nucleoli.



5



6



PLATE 2

EXPLANATION OF FIGURES

8 Adult chicken erythrocytes mounted in Tyrode solution and photographed at room temperature with ultraviolet light. Previous exposure: six seconds. After exposure the irradiation was continued till it totaled thirty-five seconds. Examined then with visible light, the cells appeared normal. $\times 1200$.

9 The same cells as those of figure 8 photographed (with ultraviolet light) after ten minutes. The increased transparency of the cytoplasm is evident. After thirty-five seconds, additional exposure to ultraviolet, the cells showed no further change.

10 Embryonic chicken blood cells photographed (with ultraviolet light) by the same technique followed for the two preceding figures. Previous exposure: ten seconds. Structure can be seen in the formed chromatin in the nuclei of both kinds of cells.

11 An adult chicken erythrocyte fixed, stained by the Feulgen reagent, counterstained with light green, and photographed using mercury light filtered through a Wratten no. 77 filter. $\times 1200$. The nuclear structure is like that to be seen in the ultraviolet pictures.

12 A macrophage from embryonic chicken spleen photographed (with ultraviolet light) at room temperature. Previous exposure: 150 seconds applied intermittently—ten-second exposures at intervals of five seconds. The cell has already begun to disintegrate; in the first photographs its outline was regular. The nucleus is faintly to be seen in the upper left-hand part of the cell. $\times 1200$.

13 Adult chicken leucocytes suspended in Tyrode solution and photographed at room temperature with ultraviolet light. Previous exposure: three seconds. $\times 1200$.

14 A macrophage from embryonic chicken spleen fixed, stained by the Feulgen reagent, counterstained with light green, and photographed as usual with mercury light filtered through a Wratten no. 77 filter. Little but the nucleus can be seen. $\times 1200$.

15 Embryonic chicken leucocytes from the slide of figure 14 fixed, stained, and photographed as before. The nuclear structure is the same as that appearing in the ultraviolet photograph of figure 13.

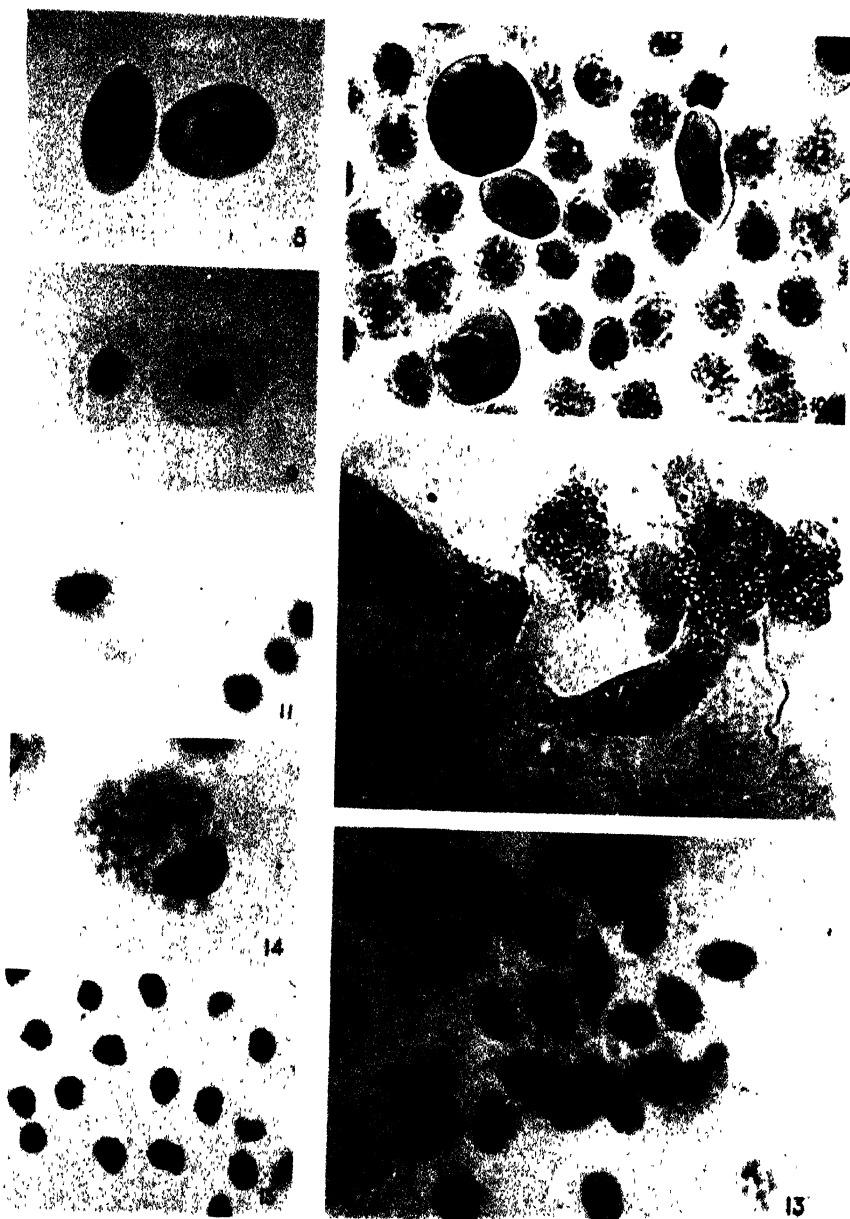


PLATE 3

EXPLANATION OF FIGURES

All of the following figures are ultraviolet photographs of fibroblasts growing in plasma. The preparations were transferred directly from the incubator to a warm stage (40°C.) of the microscope to induce cell division. The cell of the last figure is from embryonic chicken spleen, the others are from subcultures of the 'Old Strain.' The structures brought out by the Feulgen staining of dividing fibroblasts are seemingly identical with those shown in these figures.

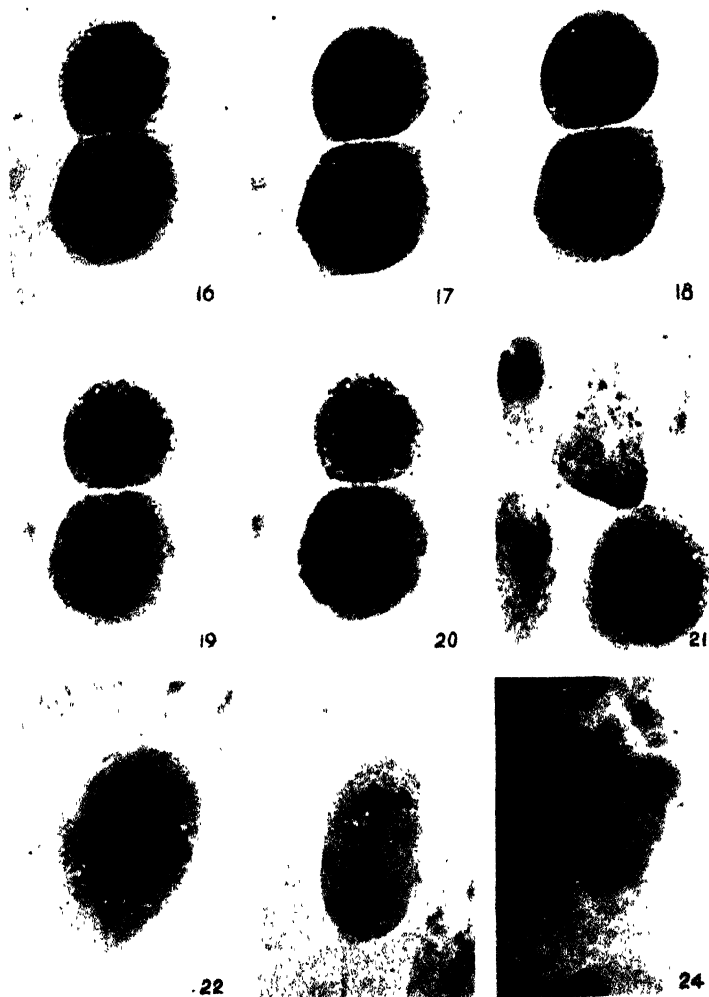
16 to 20 A series of photographs of two fibroblasts taken at intervals of from ten to twenty seconds. Total exposure: eighty-four seconds. $\times 720$. Stages in the development of the mitotic figure in the lower cell can be readily followed. The future course of the division was observed with visible light. One daughter cell disintegrated when the two cells pulled apart, the other became a resting fibroblast of apparently normal appearance. The life history of the upper cell is unknown.

21 The two cells resulting from the division of the lower fibroblast of the preceding figures. The disintegrating cell is at the top; the bottom one later became a normal resting fibroblast.

22 A cell, in metaphase, from the slide used for the preceding photographs. $\times 1200$.

23 A fibroblast, in anaphase, from the slide used for the preceding photographs. $\times 720$.

24 A dividing fibroblast from a twenty-four-hour culture of embryonic chicken spleen. $\times 1200$.



THE NUTRITION OF GLOCHIDIA DURING METAMORPHOSIS

A MICROSCOPICAL STUDY OF THE SOURCES AND MANNER OF UTILIZATION OF NUTRITIVE SUBSTANCES¹

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ONE PLATE (ELEVEN FIGURES)

AUTHOR'S ABSTRACT

Part of the nutriment of metamorphosing glochidia is supplied by the cellular host tissue, bitten by the larvae during attachment. Some of this is taken up piecemeal by the mantle cells and digested intracellularly. The coarse granules that first pack the mantle cells are apparently the precursors of a digestive secretion, some of which escapes into the mantle cavity. Here it also causes the prompt dissolution of additional utilizable host tissue.

Another source of nutriment is furnished by the provisional larval adductor muscle which undergoes degenerative changes in situ, then fragments, and finally is carried away bit by bit by amoeboid cells. These turn over their muscle content to the larval mantle, where the particles are further reduced beyond recognition. The mantle remnant itself is finally sacrificed and doubtless becomes an additional source of nutriment.

The gut serves as an organ of nutrition throughout the last two-thirds of the parasitic period. It appears to admit and digest pieces of the adductor muscle and certain unidentified particulate matter. In addition, the gut, like the definitive mantle and other organs, doubtless absorbs tissue transudate from the host.

Special vascularization of the host tissue to facilitate the passage of nutriment from host to parasite does not occur, yet there is no reason to doubt that an appreciable part of the larval nutrition results from transuding tissue juices.

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INTRODUCTORY

It is well known that the larvae of fresh-water mussels cannot metamorphose without passing a semiparasitic period,

¹Contribution no. 166. Published by permission of the United States Commissioner of Fisheries. Acknowledgment is due the staff of the Fairport Biological Station for many helpful courtesies extended during the prosecution of this inquiry. A preliminary report appears in the proceedings of the American Society of Zoölogists (Arey, '23 a).

buried in the superficial tissues of appropriate fish hosts.² This relationship between host and parasite is quite specific and each mussel has a particular host (or hosts) on which its development is restricted.

When the glochidium attaches to a gill or fin it is a tiny bivalve about one-fourth of a millimeter in diameter; the shells are lined by a simple mantle, a provisional adductor muscle closes the valves, and a small, undifferentiated mass of cells comprises the building material for the future organs (fig. 1). At the end of parasitism the juvenile mussel has not increased in gross size,³ but the internal transformation is marked. The larval mantle is replaced by a new one; the adductor undergoes destruction and two others arise de novo; in addition, there are representatives of the foot, gut, liver, heart, kidney, gills, and ganglia.

It is obvious that considerable nutriment is necessary to condition such growth. The purpose of the present paper is to inquire what the sources of the requisite food may be. Besides the academic interest in the food requirement and its supply, there is a practical bearing. The Bureau of Fisheries is attempting the rearing of mussels without parasitism; information of the sort here attempted forms a scientific basis for the intelligent conduct of such experimentation.

There are three morphological types of glochidium. The most numerous is the 'hookless' group, shaped like a boot heel or the bowl of a spoon; these are gill parasites. Next abundant are the triangular 'hooked' forms which attach to fins as well. Least common is the 'axe-head' group, gill parasites with a contour suggesting their appellation. These groups demand separate consideration.

The material studied was obtained mostly from artificial, controlled infections, samples of host tissue being removed as wanted and fixed immediately either in Zenker's or Bouin's

²In *Anodonta imbecillis* parasitism is facultative. Independent development has also been reported for *Strophitus*, but attempts to confirm this have failed repeatedly.

³Exceptions occur in the genus *Proptera*.

fluid. Paraffin sections, cut serially at 6μ and stained with hematoxylin and eosin, completed the technical procedure.

OBSERVATIONS

A. Hookless glochidia

Lampilis luteola may be chosen as a representative of the hookless glochidia. Its history throughout the period of parasitism will answer in the main for the entire group. The detailed statements that follow refer to encysted series which completed parasitism in thirteen to fourteen days on a natural host, the large-mouth black bass (*Micropterus salmoides*); this is about the average period during warm summer weather. Cysts to the number of 132 have been studied in serial section.

An account of nutrition during metamorphosis centers largely around the provisional mantle which appears to exist chiefly for this purpose. There are two mantle layers, an outer one next the shell, and an inner, thicker layer bordering the mantle cavity (fig. 1, *i.m.*, *o.m.*). The outer mantle layer is thin and inconspicuous (figs. 8 and 10, *o.m.*). It is composed of highly squamous cells, with flattened nuclei; as metamorphosis progresses, these cells become somewhat thicker and more prominent. Since they have no demonstrable connection with the problem at hand, they may be thus summarily dismissed. The inner mantle cells are of low cuboidal shape, 5 to 9μ high (figs. 1 and 8, *i.m.*). When the glochidium is attached to a gill filament, the pressure of the material bitten flattens these cells to 3 to 5μ , and their nuclei are correspondingly compressed. The nuclei are normally rounded and chromatic, with a nucleolus which does not always show prominently. Cell boundaries are not easily distinguishable, due to the granular content of the cytoplasm. These acidophilic granules, which may be observed in the living glochidium, are spherical globules between 2.0 and 3.5μ in diameter (compare fig. 3, *z.g.*). On account of their large size a few suffice to pack the cell; more will be said of them in another paragraph (p. 205). A delicate surface membrane

—unlike the striking cuticula in the hooked forms—caps the mantle cells and is continuous with the external cuticular covering of the shell (compare figs. 3 and 4, *m.c.*); thus a single cuticula bounds the glochidium inside and out (Arey, '24).

Fate of the host tissue. The amount of gill tissue bitten at the time of attachment is relatively great. Usually the glochidium's interior is filled from mantle to mantle (fig. 1, *h.t.*). In sections cut vertically through both valves, this mass measures to a maximum of $70 \times 120 \mu$. It bulks about a quarter of the cubical contents of the entire glochidium. The material bitten consists usually of epithelium, much cellular connective tissue, and an internal core of blood vessels and fibrous connective tissue. Those larvae which attach to the gill lamellae tend to be stuffed with the compressed gill layers.

Five hours after a glochidium attaches there is little or no apparent change in the mantle cells, but the cellular peripheral tissue of the bitten gill filament is variously affected. Some specimens show practically no change; in others this epithelial and cellular connective tissue loosens, the cells losing their mutual connections and becoming separate; in still others there is evidence both of tissue disorganization and disintegration.

By nine hours the soft constituents of the bitten gill may be reduced to a formless mass of débris with a pulpy, necrotic look. In some cases the dissolution is but partial, and naked nuclei are seen (compare fig. 2, *a*). The mantle cells contain pyknotic nuclei and cellular fragments; hence phagocytosis has been operative (compare figs. 2, 3, and 4). As a result of ingestion these cells increase in height, and the tallest reach 15μ . The sensory cells, having fulfilled their function when attachment occurs (Arey, '21), disappear early.

At the end of the first day phagocytosis is active. The granules of the mantle cells are plentiful and prominent, though somewhat diminished in number. Those remaining retain their original size, but stain less brilliantly than at first—a condition detectable as early as nine hours.

After two days the granules are practically gone (compare fig. 3, *z.g.*) and the nucleoli become prominent. The general cytoplasm is coarsely granular and contains miscellaneous ingested cell fragments and nuclei. At this time, and in some instances earlier, the chief cellular tissues of the bitten gill have entirely disappeared.⁴ This leaves exposed the central core of fibrous tissue and blood vessels (fig. 11, *h.t.*). In the numerous cases where the glochidium attaches to an edge of the blade-like gill filament the core is ensheathed by a dense lamina of connective tissue (figs. 10 and 11).

At five days there is evidence of the retrogression of the tougher remnant of the enclosed gill (figs. 10 and 11). By the ninth day it is shriveled, and at eleven days it is a mere fibrous strand with a few pyknotic nuclei. During the last days of encystment the persisting host tissue becomes still less conspicuous.

How are these several facts, concerning which observations have also been recorded by Faussek ('95) and others, to be interpreted? The dissolution of the cellular inclusion at five hours and its disintegration by nine seem to imply an early enzymic digestion. The granules of the mantle cells exactly resemble those which disappear from vertebrate glands during extracellular secretion. Since, moreover, the gradual loss of these glochidial granules coincides precisely with the period of reduction of the bitten host tissue, it is only logical to infer that the mantle cells also have an external digestive secretion of which the granules are the visible presecretory index. Otherwise the bitten tissue should not disintegrate so promptly, for it is easily demonstrable that excised gill filaments not only live for hours in a watch-glass, but even complete cysts of extra size about attaching glochidia (Arey, '21). Hence the bitten gill tissues, protected by an enclosing gill cyst, break down entirely too readily for the process to be

⁴ In a series of *Lampsilis anodontoides*, which completed metamorphosis on the gar-pike in six days, the bitten gill tissues were not digested until the end of the third day.

purely spontaneous. Furthermore, although phagocytosis is undoubtedly active, and cellular fragments in the cytoplasm are relatively abundant, yet the visible evidence of such ingestion is not sufficient to permit the belief that all the disappearing gill tissue is taken up piecemeal and disposed of by intracellular digestion. On the contrary, it is reasonable to conclude that part of the bitten gill tissue undergoes digestive liquefaction *in situ*,⁵ whereupon it is absorbed; in addition, many nuclei, cellular fragments, and miscellaneous débris are first ingested and then digested by the mantle cells.

There is a suggestive correlation between the tardy onset of metamorphosis and the time consumed in receiving and digesting the bitten host tissue. Structural changes in the transforming glochidium first become noticeable after the second day of encystment; during this same period the mantle is occupied with reducing the cellular tissue of the host. Does the start of metamorphic development wait upon the conversion of this material and its reception as available food? Is the initial stimulus bound up in this phenomenon? Such questions can be raised, but not answered except by inference.

Mention has been made repeatedly of the blood vessels in the center of the bitten mass. These are invariably found in well-encysted specimens whether the attachment is to the gill lamellae (capillaries) or to the blade-like edges of the filament (larger vessels). It might be thought that the blood continues to circulate in such vessels, and hence the glochidium by diffusion is supplied with a constant source of nutriment throughout its parasitic period.⁶ This possibility is particularly appealing, inasmuch as the host makes no attempt whatever to vascularize the tissues adjoining its guests (Arey, '32 a).⁷ Yet such an hypothesis is clearly untenable, for it is not supported by fact. In the first place,

⁵ Corroborative are the bare nuclei often encountered (compare fig. 2, a).

⁶ Blystad ('23-'24) has recently advanced this theory. He holds for a placental-like relation between host and parasite.

⁷ A few mussels, including the axe-head genus *Proptera*, not only metamorphose internally, but increase greatly in general body size while parasitic. I have

the adductor muscle brings the valves so nearly into apposition that the tissue included is constricted in a highly compressed fashion (fig. 11, *f*). The lumina of the vessels are thereby obliterated at this level, and the condition persists until the end of metamorphosis (Arey, '24). To state dogmatically that there can be no seepage of plasma through this isthmus is unwarranted; it conceivably may occur. But to dignify it by the term 'circulation' is to ignore the evidence of well-preserved specimens which have not been subjected to distortional violence. In any event, there is not enough passage of plasma to keep alive the host tissue beyond. The progressive involution of the fibrous core (after the digestive phase is finished) is eloquent testimony to the limited extent of such possible intravascular flow (figs. 10 and 11, *h.t.*). Still, there doubtless is fluid interchange between host and parasite, although the tissue juices which bathe the glochidium are more probably utilized for this purpose; (the gill glochidial parasite usually lies embedded in cellular connective tissue, roofed over by epithelium; Arey, '32 a). Conversely, during the extensive internal organ building of metamorphosis, as well as in the digestion of bitten host tissue and the sacrificed larval adductor, there arise katabolic wastes which demand removal; these undoubtedly follow the same diffuse path.

Involution of the larval adductor. A new and most remarkable process begins by the third or fourth day. At this time the mantle cells have lost their zymogen granules and the previously phagocytosed bits of gill tissue are digested. Now the cytoplasm again becomes coarsely and irregularly granular by the incorporation of many cellular fragments (figs. 8 and 11, *m.f.*) It is easy to overlook the discontinuity of these two ingestive and digestive periods, and fall into the error of believing that they constitute one protracted process.

observed encysted *P. laevissima* which had increased their volume some forty times. This necessitates a considerable supply of nourishment from the host. In fact, the fleshy cyst is vascularized to a slight extent, and it may be further significant that these forms alone have their valves bowed so they meet only along their ventral edges (Arey, '32 a).

What is the source of these new inclusions? In brief, the larval adductor breaks down (figs. 5, 6, and 7) and is transported bit by bit by amoeboid cells (figs. 5 to 8, *t.c.*) to the larval mantle (figs. 9 and 10, *t.c.*) which receives the fragments (figs. 7 and 10, *m.f.*) and reduces them to finer particles (figs. 8 and 11, *m.f.*) that are utilized as food for the growing organism. With the perspective gained by this general statement, the detailed history of the adductor muscle and inner mantle may now be followed.

On the third or fourth day there are indications of the future decline of the larval adductor. Some of its muscle fibers begin to swell, become somewhat opaque, and stain less brilliantly (fig. 5, *a.m.*). At the same time amoeboid cells appear among these fibers (figs. 5 to 7, *t.c.*). They have basophilic cytoplasm and vesicular nuclei; the cytoplasmic contour is irregular and often drawn out into processes. Passing alongside the swollen and fragmented muscle fibers, the wandering cells ingest irregular or rounded pieces of muscle (figs. 6 and 7, *m.f.*). Often a nest of such cells is seen in a restricted area which not always is at the periphery of the muscle as a whole (fig. 7, *t.c.*). The fragment taken in is frequently larger than the transportive cell itself (fig. 8, *m.f.*); the largest ingested piece observed in my series measured $12 \times 20 \mu$. On the other hand, a cell may contain several fragments. In such instances of marked distention it is sometimes difficult to identify a continuous cytoplasmic rim (fig. 8, *t.c.*). At other times, large fragments are surrounded by the joint effort of several fused cells. These transportive cells then move ventrad between the two mantle layers (figs. 8, 10, and 11, *t.c.*) and come to lie just outside the base of the inner mantle and in close apposition with it. Some are found pressing and indenting the mantle cell layer (fig. 9, *t.c.*). The load is then delivered to the mantle cell, but the carrier is not incorporated as well; its nucleus and cytoplasm are distinctive and do not appear within. Furthermore, unladen cells of the same type are found near the mantle and these apparently are the former transportive elements, now empty.⁸

The grosser portions of muscle become reduced promptly to smaller bits (figs. 7, 8, and 11, *m.f.*'). It is possible that this may be begun in part by the amoeboid carriers, but, even so, it is certain that many transportive cells reach the mantle with large pieces of muscle still intact (figs. 8 and 9, *m.f.*); in transit, fragments as large as $12 \times 20 \mu$ have been observed. There is some indication of a breaking down while the transportive cells lie just beneath the mantle layer. At any rate, the largest pieces of muscle seen in a mantle cell measured 10.5μ (fig. 7, *m.f.*'), whereas a diameter of even 7μ is exceptional. The average size is from 2.0 to 3.5μ .

Until the end of the eighth day, and in a few cases into the ninth day, this carriage continues. The dissolution and removal of the adductor are gradual. Portions remain unchanged until the very end of the transportive period, and, throughout the entire time, regressive and normal-appearing areas adjoin (figs. 6 and 7). Not until the eighth or ninth day do the first traces of the definitive adductor muscles appear; so it happens that the gradual, progressive removal of the larval adductor serves to provide adduction until the permanent muscles differentiate and replace it.

Attention may now be focused on the larval mantle and its content of muscle fragments. The cytoplasm, which loses its zymogen granules and early reduces the bitten gill to ordinary coarse granulations, again becomes very coarsely granular beginning with the third or fourth day (figs. 8 and 11, *m.f.*'). This is due to ingested muscle, the bits being of irregular size (2 to 3.5μ) and roughly resembling the zymogen globules now gone. However, they never stain so brilliantly; with eosin their color is a dull brownish-red and the tinctorial appearance of the mantle is flat. Incidentally, the coloration of these

* In a series of encysted *Lampsilis anodontoides*, which is parasitic on the gills of the gar-pike, and in some miscellaneous stages in the metamorphosis of *Unio gibbosus*, indications of the swelling and fragmentation of the larval adductor were likewise observed. Furthermore, in the *Unio* preparations there was not only evidence of muscle removal by amoeboid cells, but the reduced fragments were also demonstrable within the mantle cells. Thus, there is reason to believe that these processes are common for the entire hookless group.

particles and the degenerating adductor is identical and points further to their genetic relation.

By the fourth day the mantle cells are becoming tall and columnar, with rounded free ends and distinct cell boundaries (figs. 8 and 11, *i.m.*). Measurements show that this elongation is due to actual increase in bulk, and not to lateral crowding. At five days both the rounding of the free ends and the indenting at the cell junctions are pronounced; the tallest cells measure about $27\ \mu$. Until the end of parasitism conditions remain much the same. The cells attain a maximum height of $30\ \mu$ and the nucleus lies below the middle level (compare figs. 8, 10, and 11). Toward the free surface the cytoplasm is densely packed and granular; it stains dully—a brownish-orange with eosin. The basal half of the cell is vacuolate, or clear, and is often crossed with reticulate strands (fig. 10). After the eighth day, pieces of muscle as distinct entities are usually no longer seen in the cytoplasm, although exceptions occur.

As metamorphosis proceeds, the larval mantle⁹ is crowded toward the center of the valve by the developing permanent mantle which encroaches from below and on both sides (figs. 10 and 11, *d.m.*); it does not grow ventrad from the region of the gill buds, as in Anodonta. This reductional process is evident from the seventh day on. There is no mistaking the limits of the two mantles, for each is distinctive. The definitive one has shorter, columnar cells with the nuclei at the free ends, surrounded by dark basophilic cytoplasm; the basal two-thirds or more of the cell is clear and pale. In the early days of their appearance these cells are cuboidal, but the same characteristics dominate; throughout the parasitic period the cells are remarkably regular and block-like, and their boundaries are exceptionally distinct.

Toward the end of parasitism the larval mantle decreases in height and its cells then range from 9 to $18\ \mu$. This diminution possibly may be due in part to pressure from the grow-

⁹ This is homologous with the so-called mushroom body, found in the transforming hooked glochidium.

ing foot, but in addition there is doubtless some independent shortening. They also lose part of their distal granulation, and the cell walls again appear less distinct. Nevertheless, there is no general cell fusion before the metamorphosing mussel becomes free. Concerning the ultimate disappearance of the larval mantle I can make no statement except that it is retained for a period by the free-living juvenile.¹⁰ This is an unexpected discovery, for in the hooked forms, to which attention has hitherto been confined, it is known to vanish toward the end of parasitism.

As the foot gains prominence, beginning with the seventh or eighth day, it comes in apposition with the larval mantle, and, with continued growth, presses against it. For the last third of the parasitic period the two are largely in contact. It might be suspected that the distally stored nutriment of the mantle is transferred in a soluble condition to the foot and thence becomes distributed to the growing organism. Although the foot is held to be absorptive in adults (Churchill, '16), this particular transfer in the larva cannot be detected microscopically by ordinary methods.

It is perhaps worth while to point out that the larval mantle, although ectodermal in origin, is involved in various nutritive functions which usually characterize the entodermal gut of animals. The somewhat similar adaptation of the ectodermal mammalian placenta is brought to mind.

Nutritive activities of the gut. The enteric canal becomes established as a tube at about the end of the first third of parasitism. What part, if any, does it take in the nutrition of the metamorphosing larva?

At the fifth day of my series the stomach contains material that stains precisely like the degenerating adductor muscle. It tends to be relatively bulky and to conform to the shape of the lumen, like a cast. In all the specimens examined this

¹⁰In *Lampsilis anodontoidea* the mushroom-shaped mantle likewise is still prominent at the end of parasitism. In *Proptera laevis*, which is exceptional in that it continues to grow on the gills for some time after metamorphosis, stages were observed which had increased forty-fold in volume yet retained a typical 'mushroom body.'

content had undergone partial digestive resolution, and accordingly had lost the distinctive character of muscle; this is also true of the disrupted adductor itself which is homogeneous in the same preparations. The presence of this eosinophilic mass antedates the formation of the coarse liver granules of similar stainability. It, therefore, is not a coagulum of liver secretion.¹¹ Rather than urge this interpretation it would be more logical to assume the reverse and make the stomach content the genetic antecedent of the liver granules, or at least of their eosinophilic component; yet I have no inclination to do so. From these findings, meager as they are, one can infer that a certain fraction of the provisional adductor is received into the developing alimentary tract and digested there.

Especially in the second half of the parasitic period, finely particulate material of uncertain origin is present in the enteric tube. It seems reasonable that tissue transudate from the host is taken up by the gut, but as to other specific elements there is no clue. The larval mantle, although atrophic, lasts beyond the period considered here. There is nothing in the stages examined to indicate its direct reception into the digestive canal.

Such facts seem to indicate that the gut is functional as a digestive organ during two-thirds of encystment. Its rôle may well be important, even though the visible evidence for this is slight and bespeaks rather a subordinate activity.

B. Hooked glochidia

If attention be now directed to the group of hooked glochidia, many comparable conditions are found. My observations are based chiefly upon a series of infections of *Anodonta corpulenta*, attached to the fins of the orange-spotted sunfish, *Lepomis humilis*, and upon other infections, both artificial and natural, of *Hemilastena ambigua* on the gills of its urodele host, *Necturus maculatus*. The *Anodonta* infection was carried out at a temperature of about 20°C.

¹¹ Toward the end of parasitism, however, large liver granules do find their way into the adjoining gut lumen.

The *Hemilastena* series were procured during colder weather, in the middle of October. These thermal differences must be borne in mind with respect to the time factor in order to reconcile the descriptions which follow with those of the preceding pages.

Fate of the host tissue. In *Anodonta* the bitten fin is locked firmly between the flexed valvular beaks and their stout spines (Arey, '24). This host material consists of epithelium several layers deep, and usually includes some of the firmer core of the fin, such as connective tissue, blood vessels, and fin rays. The amount is about half that taken by *Lampsilis luteola*. Similarly, *Hemilastena* bites on the stratified epithelium of the gill and, usually, on connective tissue and capillaries as well (fig. 2, *h.t.*).

In each, the mantle proper consists of large cuboidal cells, packed with acidophilic granules (figs. 2 and 3, *z.g.*); although coarse (1μ), they are but one-third to one-half the diameter of the corresponding granules of *Lampsilis luteola*. At first the cell boundaries are indistinct, but sometimes they are clearly discernible; this masking is due to the zymogen granules. The nuclei are vesicular and contain a prominent nucleolus. The free surface, next the mantle cavity, bears a prominent cuticular membrane (figs. 3 and 4, *m.c.*); in *Hemilastena* it shows vertical markings, like canaliculi, which give the whole the appearance of a row of blocks. This membrane is much heavier than the delicate one, scarcely distinguishable, in *Lampsilis luteola*.

Shortly after encystment the zymogen granules begin to disappear from the mantle cells. In some specimens of *Anodonta* they had vanished completely by thirty-six hours; in others there was great depletion, but not total loss, at that time. Cell boundaries now show plainly; the cytoplasm is vacuolate and stains without brilliancy. In *Hemilastena* some mantle cells may lose all their granules while adjoining cells are intact (figs. 2 and 3); there is also a marked tendency for the distal granules to disappear before the basal ones do (fig. 2); the cytoplasm then appears slightly basophilic and the cell boundaries distinct.

While these changes have been taking place in the mantle, there is modification of the ingested mass as well. In *Hemilastena* the epithelium rounds up and separates from the connective tissue, still held by the hooks. The epithelial elements bitten by *Anodonta* begin to loosen and disintegrate soon after encystment. Apparently there is digestion in the mantle cavity, coincident with the loss of mantle-cell granulation; this is illustrated by the bare nucleus, *a*, in figure 2. At thirty-six hours, in the *Anodonta* series, all but the more resistant fibrous tissue or fin rays may be largely gone, and by the second day this dissolution of the softer elements is complete. The firm residue of tough tissue remains for the most part undigested and with only partial further reduction throughout the metamorphic period.

Besides the probable extracellular disintegration and digestion in the mantle cavity there is phagocytic ingestion by the mantle cells. *Hemilastena* demonstrates this clearly because of the huge size of the *Necturus* cells. In figure 2 a fortunate cut brings three stages of the process within the limits of a single section; at *a* is a nucleus, denuded of its cytoplasm, in contact with the mantle; at *b*, the cytoplasm of the mantle cell is protuberant and fused with that of a host cell. Stage *c* shows a cell completely ingested. As such cells are large they may equal or exceed the bulks of their captors; for example, cell *a*, figure 3, has a diameter of $13\ \mu$. At *b* of the same figure is an ingested pigment cell. Similarly, in *Anodonta*, twenty-nine hours after encystment, the mantle contained much host material; these pieces measured to $9\ \mu$ in diameter.

It is important to inquire how ingestion occurs through such heavy cuticulae. Preparations were found in both *Hemilastena* and *Anodonta* which showed cells being taken in through breaks in the membrane (fig. 4, *a*). There was no appearance of artefact in these openings, and it must be the regular method of entrance, since the material obviously passes through in some manner. Just how the cuticula is opened would be highly instructive, but to this there is as yet no clue.

During the first two days of parasitism ingestion is steadily going on in Anodonta. The fragments and particles thus received are stored partly in vacuoles and partly in the compact cytoplasm. By thirty-six hours the vacuoles are abundant and may be huge, apparently by coalescence; they occupy much of the cell's base and contain one to several fragments. Smaller vacuoles are also present in the upper part of the cell. What is their significance? Are they like the food vacuoles in a protozoon, or are they caused by a liquefaction of the foreign tissue? In any event they are transitory—at least in their exaggerated expression; at seventy-two hours only small vacuoles persist, giving the cytoplasm an alveolar appearance.

During this period of ingestion the mantle cells increase in size and become taller as the result of food received and digested. The mantle cells of Anodonta put out elongate conical or finger-like processes which are often local and do not involve the whole surface of a cell. They differ from typical pseudopodia, inasmuch as a cuticula covers them. It does not seem probable that they result from crowding by the new mantle; their shape and distribution negative a mere mechanical distortion. These peculiar extensions are present while ingestion is going on.

The mushroom body. The incorporated host tissue is largely digested by the third day in Anodonta. By this time the so-called mushroom body has put in its appearance. It is nothing else than the larval mantle, forced toward the center of the valve both by the definitive mantle cells which creep centripetally from the periphery and by the gill buds and more of the mantle which likewise crowd ventrad from above. The larval mantle thus progressively becomes limited to the central regions of the valve and gradually assumes a pedunculated mushroom appearance. The component cells are tall ($15 \times 45 \mu$ in vertical section), granular, vacuolate (especially at the base), and poorly acidophilic; their nuclei are vesicular, with a prominent nucleolus, and are located basally. At seventy-two hours the cells are still separate, though they

soon fuse. The free surface of the mushroom body extends in rounded cell tips, usually deeply notched at the cell junctions; this gives an irregular scalloped contour. Is the mushroom appearance caused by pressure from all sides? Probably in part—especially the constricted base—but the cells have also increased greatly in volume since encystment, as direct measurements show. The mushroom body is gradually reduced, and toward the finish of the parasitic stage it no longer is recognizable.

Involution of the larval adductor. The larval adductor is destroyed about the middle of parasitism. This is at the time when the mushroom formation is most pronounced. Although properly graded older stages were not available, indications in miscellaneous sections of other encysted hooked glochidia point to a later history similar to that already described for the hookless group. Furthermore, portions of the adductor are demonstrable within the mushroom body. It would thus seem that both mushroom body and adductor serve as food, and the former, although steadily regressing, is useful in the glochidial economy longer than has generally been supposed. During its decline it is still active in reducing the enclosed muscle fragments to more easily utilizable material.

While the involution of the mushroom body advances, the definitive mantle is growing correspondingly. On their first appearance its cells are short columnar to cuboidal in shape. The cytoplasm is basophilic and the nuclei stain so heavily as to mask the nucleoli.

SUMMARY AND CONCLUSIONS

The glochidia of fresh-water mussels undergo an internal metamorphic development while encysted upon appropriate aquatic vertebrate hosts. This growth and differentiation can proceed only when certain environmental requirements are fulfilled. Concerning some of these factors nothing is known, and of others there are mere intimations. Thus, it is sufficiently established that the parasitism is highly specific and demands definite hosts. Enough concerning the natural im-

munity of non-hosts and the acquired immunity of normal hosts has been discovered to show that delicate adjustments and relations exist (Arey, '23 b, '32 b); but as to the actual nature of the immunity, or even the factors involved, next to nothing is known. Again, it is apparent that a favorable proximity to the available oxygen supply is furnished by the superficial, ectoparasitic location of the larva. That the oxygen potential, even on gill filaments, is concerned with the inception or progress of metamorphosis seems reasonable, and even probable, yet such remains unproved.

On the other hand, a considerable amount of nutriment is surely necessary to condition the rather extensive development and differentiation that characterize metamorphosis. The sources of this raw nutritive material are several: The host tissue, bitten during attachment, supplies part; the larval adductor muscle is sacrificed as an additional supply, and the same is probably true of the atrophying mushroom body; direct transudation of the host's tissue juices doubtless accounts for the remainder.

The cellular host tissue enclosed between the valves of an encysted glochidium is removed in two ways. A portion is taken up by the cuticular-covered mantle cells and digested intracellularly. Further, there is sufficient reason to believe that the coarse granules that first pack the mantle cells are the precursors of a digestive secretion, which, at least in part, escapes into the mantle cavity; here it acts on much of the host tissue, causing its prompt dissolution and resolution into utilizable fluid nutriment.

Another source of nutriment is furnished by the provisional larval adductor muscle which undergoes degenerative changes in *situ*, then fragments, and finally is carried away bit by bit by amoeboid cells. These turn over their muscle content to the larval mantle, where the particles are further reduced beyond recognition.

The larval mantle increases greatly in thickness, but steadily diminishes in area, as the definitive mantle grows centripetally to replace it. In the hookless glochidia the larval

mantle, or 'mushroom body,' persists until after the end of parasitism; in the hooked forms it disappears toward the end of the parasitic period.

In general, the provisional mantle serves first as the bearer of sensory cells which are instrumental in securing attachment to a host (Arey, '21), and then for the dissolution and ingestion of the bitten host tissue. Next, it receives and reduces the degenerating adductor. Finally, the mantle remnant is sacrificed and doubtless serves as an additional source of nutriment.

Special vascularization of the host tissue to facilitate the passage of nutriment from host to parasite does not occur (Arey, '32 a), yet there is no reason to doubt that an appreciable part of the larval nutrition results from transuding tissue juices. This belief is strengthened by the apparent necessity for the removal of katabolic wastes along the same path but in the reverse direction. Since the internal transformation of a glochidium is relatively extensive, its metabolic slag surely does not remain at the site of origin.

The gut serves as an organ of nutrition throughout two-thirds of the parasitic period. It appears to admit and digest part of the adductor muscle and certain unidentified particulate matter. In addition, the gut, like the definitive mantle and other organs, doubtless absorbs tissue transudate from the host. What proportion of the total food is cared for by the gut cannot be determined at present.

These results supply information as to the food requirements of transforming mussels which should be considered in future attempts to rear commercial shells by culture methods in the absence of parasitism.¹²

¹² In the continuance of such experiments it may prove worth while to consider whether the epithelially embedded fin parasites, robust in structure and less intimately associated with the hosts' tissues, are not the most favorable type of experimental material.

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PLATE 1

EXPLANATION OF FIGURES

All the figures are photomicrographs of vertical sections cut dorsoventrally through both valves of the glochidia.

1 *Lampsilis luteola* completely encysted after an attachment of two and one-quarter hours on a gill filament of *Micropterus salmoides*. $\times 150$.

2 *Hemilastena ambigua* encysted two and one-quarter days on the gill of *Neecturus maculatus*. Three successive stages in the ingestion of red blood cells by the mantle are shown at *a*, *b*, and *c*. $\times 355$.

3 *Hemilastena* encysted one day. The mantle has ingested a red blood cell (*a*) and pigment masses (*b*). $\times 710$.

4 *Hemilastena* encysted one day. The mantle is receiving a blood cell of the host (*a*) through a gap in the prominent cuticula. $\times 710$.

5 *Lampsilis luteola* encysted six days. The initial swelling of the larval adductor fibers is shown. $\times 355$.

6 *Lampsilis luteola* encysted five days. Partial dissolution of the adductor and the arrival of transportive cells are illustrated. $\times 355$.

7 *Lampsilis luteola* encysted four days. A nest of muscle fragments and transportive cells show. $\times 355$.

8 *Lampsilis luteola* encysted four days. Transportive cells with muscle fragments are passing between the two mantle layers. $\times 700$.

9 *Lampsilis luteola* encysted seven days. A transportive cell and its contained muscle fragment presses against the inner mantle layer. $\times 710$.

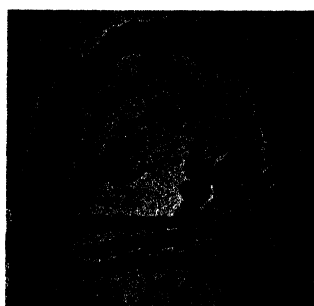
10 *Lampsilis luteola* encysted seven days. The transportive cells and larval mantle show. $\times 355$.

11 *Lampsilis luteola* encysted five days. The larval mantle contains many adductor fragments. $\times 355$.

ABBREVIATIONS

a.m., adductor muscle
a.m.', swollen adductor fibers
c.w., cyst wall
d.m., definitive mantle
f. flange
h., hinge
g.f., gill filament
h.t., host tissue

i.m., inner mantle cells
m.c., mantle cuticula
m.f., muscle fragments
m.f.', muscle fragments in mantle
o.m., outer mantle cells
s., shell cuticula
t.c., transportive cells
z.g., zymogen granules



THE ARCHITECTURE OF THE PARIETAL CELLS OF THE SALIVARY GLANDS OF THE GRASSHOPPER, WITH SPECIAL REFERENCE TO THE INTRACELLULAR CANALICULI, GOLGI BODIES, AND MITOCHONDRIA

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THREE PLATES (TWENTY-FOUR FIGURES)

AUTHORS' ABSTRACT

The parietal cells of the salivary glands of the grasshopper are large and roughly spherical or sometimes pyramidal in form, scattered for the most part singly among the zymogenic cells of the lobules. They possess a magnificent system of intracellular canaliculi into which their secretions are directly emptied and conveyed to the exterior of the cell. These intracellular canaliculi are strikingly impregnated with osmic-acid methods (Nassonov, Mann-Kopsch) in a manner comparable to the contractile vacuole of *Paramecium* as described by Nassonov. However, in addition to the impregnated system of intracellular canaliculi, Golgi bodies are found evenly distributed throughout the cell in the form of crescents, semicircles, or ring-like structures typical of insect tissue.

Inasmuch as a direct homology between the intracellular canaliculi and the contractile vacuole of *Paramecium*, both from the point of view of morphology and physiology, seems justified, we question the inference drawn by Nassonov that the impregnated walls of the contractile vacuole in *Paramecium* actually represent Golgi material. Deduction is made from these and previous studies that the magnificent network of 'vacuome-Golgi apparatus' described by Parat and Painlevé ('24) in the salivary glands of *Chironomus* larvae is nothing more or less than intracellular secretory canaliculi.

Mitochondria in the parietal cells are abundant in the characteristic form of short rods and filaments. No evidence of a direct transformation of mitochondria into Golgi bodies, as held by Parat, was observed. Golgi bodies and mitochondria show no marked topographical relationship to the intracellular secretory canaliculi.

Among the problems under discussion in modern cytology, none is of more general interest than that of the Golgi material. Parat and Painlevé have interpreted certain vacuoles, which stain with neutral red in the salivary glands of *Chironomus* larvae, as the Golgi apparatus because at certain stages these vacuoles seem to fuse to form a network which may be impregnated with silver. Krjukowa holds that this impregnated network really represents intracellular secretory canaliculi which may be filled with secretion products. Such intracellular canaliculi have been described in a large number of cells, especially those of mammals, insects, crustacea, and annelids, but there is a general tendency to believe

that in many cases they do not represent permanent structures which open to the exterior of the cell. In addition, it has been assumed that the Golgi material has a direct morphological connection with the origin of secretions. Such a view is also held with regard to the mitochondria. Whatever the ultimate decision in regard to these matters, the distribution of the Golgi material, of mitochondria, and of secretory granules in relation to that of intracellular canaliculi should be of importance. Further, since the lipoid membrane of the contractile vacuole of certain ciliates may be impregnated with osmic acid and is obviously secretory in nature, Nasonov has interpreted it as homologous to the Golgi material.

It is hoped that the observations recorded in this paper may throw some light on the problems briefly outlined above.

MATERIAL AND METHODS

The material for this study consists of certain cells of the salivary glands of grasshoppers (*Rhomalea microptera*, *Melanoplus differentialis*, and *Melanoplus femur-rubrum*) which have proved excellent material for the demonstration of intracellular secretory canaliculi. Some of the tissue was prepared according to the techniques of Nasonov and of Ludford for the demonstration of the Golgi bodies; these methods also demonstrate the intracellular secretory canaliculi. They were also used by us to disclose the lipoid membrane of the contractile vacuole in *Paramecium caudatum* and certain other ciliates.

Other salivary glands were prepared by the mitochondrial method of Regaud, and by the method of Delaney ('27). To reveal the 'vacuome' of Parat 1 per cent neutral red dissolved in physiological salt solution was injected into the body cavity of the grasshopper and after several hours the salivary glands were examined mounted on slides in physiological salt solution. Other salivary glands were removed from grasshoppers and placed in a weak solution of neutral red in physiological salt for varying lengths of time. Still other glands were examined supravivally without staining,

and the intracellular canaliculi could be seen almost as clearly as in the fixed and stained preparations.

DESCRIPTION

The salivary glands of the grasshopper are paired organs which are embedded in the connective tissue of the ventral surface of the thoracic cavity. The glands are of the racemose type and are composed histologically of at least two distinct types of secretory cells, which we have proposed to call zymogenic and parietal. It is with the latter of these cells that we are here concerned.

The parietal cells are large, roughly oval or pyramidal in form, and possess, as a rule, a single nucleus which is embedded in a homogeneous or finely granular, slightly acidophilic cytoplasm. The position of the parietal cells in the lobule of the gland is somewhat variable, but is, in a general way, comparable to that of the parietal cells of the stomach of mammals; that is, they are scattered for the most part between the zymogenic cells. In some cases they are found in groups of three or four cells (figs. 5 to 12), in which case their efferent ductules usually open into a common duct or reservoir. Generally, the broad base of the more or less triangular body rests in direct contact with the basement membrane, the wide tip wedged between the bases of adjacent zymogenic cells (figs. 13 to 22). Although the parietal cells have been forced away from direct contact with the lumen of the gland, they nevertheless possess a direct communication with it by way of inter- and intracellular canaliculi (fig. 14, figs. 16 to 22). It is this magnificent system of intracellular canaliculi that is perhaps the most remarkable and typical feature of the parietal cells (figs. 1 and 15). The intracellular canaliculi appear in the living non-stained cell as a system of ductules of considerable variation in degree of complexity, often containing secretory material, and occupying the central area of the cell between the nucleus and cell periphery. In the parietal cells of the salivary glands of *Melanoplus differentialis* (fig. 2), the intracellular cana-

liculi are much simpler than those of the parietal cells of *Rhomalea microptera* (fig. 1). The intracellular canaliculi of the parietal cells of *Melanoplus differentialis* often present a Y-shaped appearance, with the forked portions of the Y extending well around the sides of the nucleus to end blindly in the region of the base of the cell. The forked canaliculi unite at the apex in the luminal end of the cell to form in most cases a single large efferent ductule which becomes continuous with the intercellular canaliculi and hence with the lumen of the gland. In other cases the forks of the Y give rise to several branches, all of which help to collect the secretory material from the cytoplasm of the cell.

In the parietal cells of *Rhomalea microptera* is found a system of intracellular canaliculi that is much more complicated than that of *Melanoplus differentialis* (fig. 1). In fact, the intracellular canaliculi thoroughly penetrate the cytoplasm and display a magnificent system of branches giving rise to a complicated net-like structure (figs. 13, 14, 15). The increase in degree of complexity of the canaliculi seems to be directly associated with the increase in size of the cell. In many cases secretion droplets are found lying within the canaliculi or in close proximity to them. They seem to form at practically any position in the cell and are emptied directly into the canaliculi at the nearest point. The secretion stains readily with neutral red and apparently enters the canaliculi either as formed droplets or as a fluid stream; in any case, it gives rise, when present in sufficient quantities, to a continuous mass of secretion in a way similar to that described by Parat and Painlevé ('24) for the salivary glands of *Chironomus* larvae and by Saint-Hilaire ('27) for the silk glands of hymenopterous larvae.

There has been much discussion as to the relation of the intracellular canaliculi to the surface of the cell. However, the demonstration of them in the living unstained cell and in ordinary stained preparations seems to have left little doubt as to the presence of a ductule opening directly to the surface of the cell. The canaliculi are beautifully demonstrated by

osmic-acid impregnation methods (Nassonov, Ludford), in which they stand out as definite canals with deeply impregnated walls. The impregnation of the walls is, we believe, a similar phenomenon to that which takes place in the blackening of the walls of the contractile vacuole in certain ciliates.

The blackening of the walls of the intracellular canaliculi by osmic-acid methods brings up the question of the nature of the limiting membrane of the intracellular canaliculi. In preparations that have been stained by Regaud's method a definite outline of the membrane of the intracellular canaliculi is discernible. In fact, this membrane seems to be somewhat thicker than either the membrane of the nucleus or that of the cell. Its deep impregnation with osmic acid suggests an interpretation comparable to that advanced by Nassonov ('24) for the contractile vacuole of *Paramecium*, i.e., that the limiting membrane is of lipoidal nature (figs. 23 and 24). However, our observations do not substantiate the hypothesis of Nassonov that this limiting membrane represents the Golgi material, for in preparations which show beautifully the impregnated canalicular lipoid membrane, the typical insect Golgi bodies may also be found (fig. 3). The Golgi bodies possess their characteristic form and are made up of two components (Golgi complex), an osmiophilic cortex with an osmiophobic medulla. They are fairly constant in number and seem to be evenly distributed throughout the cell. That is, they show no apparent concentration about the intracellular or intercellular canaliculi, as seems to be the case for the Golgi material in the liver cells of certain amphibians (Makarov, '30; Dornesco, '31; Pollister, '31); neither do they display a constant relationship to the developing secretory inclusions. To be sure, in many cases the Golgi bodies may present a close topographical position to the secretory inclusions, but in many other cases no direct relationship between the two is revealed. Therefore, we are not sure that the close association of the Golgi bodies to the secretory droplets in some instances actually signifies a physiological association or is simply a fortuitous position. While the conclusion

that secretory products have their origin from the Golgi material is still a debated question, we cannot but feel that this conclusion has in many cases been overemphasized in the current cytological literature.

In preparations of the parietal cells made according to the method of Regaud ('10), one finds that the mitochondria are abundantly present (fig. 4). They appear in the characteristic forms of granules, short rods, and rather long filaments. Like the Golgi bodies, they are found distributed fairly evenly throughout the cell. There are many more mitochondria than Golgi bodies, and it seems altogether likely that the entire volume of the mitochondria would greatly exceed that of the Golgi bodies. Yet, notwithstanding the copious presence of mitochondria in the parietal cells, no observations were made which might indicate that they are directly associated with the formation of the secretory products.

Much discussion of late has centered around the problem as to the possible relationship of mitochondria to Golgi bodies. Parat and his associates have revived the view long ago suggested by Benda (Gatenby, '31), that the mitochondria give rise directly to the Golgi bodies ('active' chondriosomes). In fact, Hosselet ('31) in a very recent memoir which contains observations on the mitochondria of many types of insect tissues has given new impetus to this idea. However, with this view we cannot agree, and our observations uphold the classical interpretation that the Golgi bodies and mitochondria are discrete bodies. Hence, as regards this question we can immediately say that there may be a transformation of mitochondria into Golgi bodies, but we feel convinced that such a relationship has not been demonstrated.

DISCUSSION

It has been vigorously urged by Holmgren that intracellular nutritive canals (Saftkanälchen) which open at the periphery of the cell are present in practically every type of cell—a view which has not been widely accepted. As a matter of fact, Holmgren's descriptions of the trophospongium and

Saftkanälchen have been quite generally interpreted by cytologists as simply the positive (trophospongium) and negative (Saftkanälchen) images of the apparatus of Golgi (Cajal, von Bergen, Cowdry, Bensley, Bowen, Ludford, Gatenby, Beams, and others). However, the extension of the intracellular canaliculi (negative image of Golgi apparatus) to the surface of the cell as claimed by Holmgren has been denied by most cytologists.

It will be recalled, however, that intracellular canaliculi with a definite efferent ductule opening to the surface of the cell have frequently been described in certain of the gland cells of insects (Gilson, '88; Dierckx, '99; Leydig, '91; Casper, '13; Saint-Hilaire, '27; Krjukowa, '29; Marshall, '30, and others). In like manner, intracellular canaliculi have been reported for the hepatic cells of the liver, although their presence is by far from being an established fact (Maximow, '30). However, the presence of such canaliculi in the parietal cells of mammals as described by Müller ('92) seems to be of less questionable nature. In fact, the important work of Harvey and Bensley ('12), who discovered that a number of vital dyes when injected intravascularly in isotonic salt solution stained all of the secretion within the canaliculi of the parietal cells, seems to be sufficient to establish their reality.

This brief survey of the literature clearly indicates that the presence of intracellular canaliculi has been described here and there in certain more or less limited and 'special' types of cells. But the lack of their general occurrence in gland cells and the apparent misconception of them by Holmgren in many cases at least, seem to have caused considerable skepticism among many cytologists as to their reality, accordingly depriving them of the place they well deserve in cytological literature.

It will be recalled that Parat and Painlevé, working "*sans aucune idée préconçue*" on the salivary glands of *Chironomus* larvae, reached the remarkable conclusion that "*. . . dans les cellules des glandes salivaires des larves du Chironome l'indépendance du chondriome et du vacuome, et nous avons*

pu assimiler á celui-ci, au moment de l'excrétion, l'appareil réticulaire interne de Golgi et, pro parte, l'appareil trophospongiol de Holmgren." In other cases they say "... lorsqu'un réseau a été décrit, il s'agit d'une confluence artificielle de vacuoles due à l'action du fixateur et renforcée par le dépôt d'argent ou d'osmium." All of which led them to the important conclusion that "Il est néanmoins certain que la 'trophosponge' (by which they also mean the Golgi apparatus) résulte d'une déformation considérable de l'appareil vacuolaire et qu'elle est bien plus un artefact que l'image de la réalité."

This rather surprising conclusion of Parat and Painlevé was received with considerable favor by several prominent histologists and embryologists; but it was never convincing to those who were intimately familiar with the form of the Golgi material in insect tissue. In fact, the description by Parat and Painlevé of the Golgi material as such a voluminous network in insect tissue immediately aroused skepticism as to their interpretation which led hastily to a reinvestigation of *Chironomus* salivary glands. Krjukowa ('29) and Beams and Goldsmith ('30) have gone over the same material and they agree that it offers no exception to the presence of typical insect Golgi bodies. This makes it seem very likely that Parat and Painlevé never saw the Golgi material at the time of their original publication. The suggestion was made by Krjukowa and by Beams and Goldsmith that what Parat and Painlevé were describing as Golgi apparatus simply represents secretory material. The massive network which they figure following staining by neutral red probably represents the fluid secretion in the net-like intracellular canaliculi. In cases where they have blackened the canaliculi by metallic compounds it is probably due to an impregnation of the secretory material plus the semipermeable membrane in the walls of the intracellular canaliculi. In fact, the present investigation together with that of Saint-Hilaire ('27) and Krjukowa ('29) beautifully substantiates the above view and offers a clear understanding as to how Parat and Painlevé were led

to their erroneous conclusion in *Chironomus* salivary glands. Saint-Hilaire, for instance, has shown in an important recent work, apparently without knowledge of the findings of Parat and his associate, that intracellular canaliculi become filled with secretory material stained with neutral red, and give rise to a massive red net-like structure similar in every way to the 'vacuome-Golgi apparatus' of Parat and Painlevé (compare figs. 23, 24, 34, 43, and 50 of Saint-Hilaire with figs. 5 and 8 of Parat, '28). In like manner, Harvey and Bensley ('12) have also demonstrated that neutral red stains the secretion within the intracellular canaliculi of the parietal cells of the stomach, thereby disclosing beautifully their morphology.

While we have no such complete series of observations as collected by Saint-Hilaire, we have been able to confirm his results in our material in a general way. Furthermore, intracellular canaliculi of the parietal cells are readily impregnated by osmic acid, all of which further adds to the already convincing interpretation that Parat's net-like 'vacuome-Golgi apparatus' represents only fluid secretion within the intracellular canaliculi. Had Parat and Painlevé been familiar with the literature on the intracellular canaliculi of the glands of insects and also with the Golgi material in insects, perhaps they would not have arrived at such a conclusion. It would seem, therefore, that the observations of Krjukowa ('29), Beams and Goldsmith ('30), and Saint-Hilaire ('27), together with the observations recorded in this paper, clearly explain step by step the faulty reasoning of Parat, and we believe these observations will go a long way in laying at rest Parat's hypothesis that the neutral-red 'vacuome' is the Golgi apparatus.

The observations recorded in this paper seem to warrant a direct comparison to the conclusion of Nasonov ('24) that the lipid membrane surrounding, or in close proximity to, the contractile vacuole and its associated canals of certain ciliates is homologous to the classical apparatus of Golgi.¹

¹ Sanchez and Cajal apparently had previously suggested this homology (Holmgren, *Anat. Anz.*, Bd. 46.

Nassonov arrived at this conclusion not merely upon the fact that the contractile vacuole impregnates with osmic acid, but also upon the morphology, and perhaps most important of all, upon similarity in function to the Golgi apparatus. It will be recalled that Nassonov in his earlier work urged the view that the Golgi apparatus is of lipoidal character and that its function is chiefly that of synthesizing the secretory material. In his later work he goes one step further and suggests that the secretion of fluid into the contractile vacuole is an exactly comparable process to that of the formation of secretion in typical gland cells. Accordingly, the dark-impregnated semipermeable membrane which is embedded in the wall of the contractile vacuole and associated feeding canals of *Paramecium caudatum* is thought by Nassonov to perform the function of secretion from the cytoplasm into the contractile vacuoles. This view of Nassonov's has been rather widely accepted by biologists, as the following quotation from MacBride and Hewer ('31) will indicate. They say:

It may be taken for granted therefore that in those protozoa possessed of a contractile vacuole the Golgi apparatus of the metazoa is represented by a lipoidal substance in the neighborhood of the wall of that vacuole. This homology, important as it is from the morphological aspect, is doubly so from the physiological or functional point of view.

To turn now to a critical comparison of the contractile vacuole of *Paramecium* with the intracellular canaliculi, we find that they both represent intracellular secretory canals. Both are surrounded by an osmiophilic semipermeable membrane which serves the important function of permitting their secretions to pass out of the cell; all of which supports the conclusion that in both cases we are dealing with structures that are comparable from both the morphological and physiological points of view. If this interpretation is correct that the osmiophilic membrane surrounding the contractile vacuole in *Paramecium* is comparable to the osmiophilic membrane surrounding the intracellular canaliculi of the parietal cells of the salivary glands of the grasshopper, it seems

highly improbable that Nasonov's theory is correct; for it has been clearly demonstrated that the osmiophilic membrane surrounding the intracellular canaliculi of the parietal cells does not represent the Golgi material. Then, too, as has been pointed out by numerous investigators, the nuclear membrane often impregnates in a manner comparable to the osmiophilic membrane surrounding the contractile vacuole. Besides, Poisson finds in *Anophrys sarcophaga* and Moore in *Blepharisma undulans* that the contractile vacuole is not impregnated, but that there are present small osmiophilic globular bodies distributed through the endoplasm which might possibly be homologized with the Golgi bodies of Metazoa.

The evidence presented in the foregoing discussion tends to support the conclusion arrived at in this paper that the lipid membrane surrounding the intracellular canaliculi is not comparable to the classical apparatus of Golgi. Accordingly, by inference, strong evidence is presented against the Nasonov hypothesis.

CONCLUSIONS

1. There exists in the parietal cells of the salivary glands of the grasshopper a much branched system of intracellular canaliculi which opens directly to the surface of the cell by way of an efferent ductule. The efferent ductule becomes continuous with the intercellular duct that joins the lumen of the gland. The degree of complexity of the intracellular canaliculi seems to be directly associated with the size of the cell.

2. The intracellular canaliculi with their associated limiting osmiophilic membranes are directly comparable both from a morphological and physiological point of view to the contractile vacuole and associated osmiophilic membrane of certain ciliates as described by Nasonov. However, besides the osmiophilic limiting membrane (Golgi apparatus of Nasonov) there are present in the parietal cells typical insect Golgi bodies evenly distributed throughout the cell. Accord-

ingly, the inference is drawn that the lipid membrane surrounding the contractile vacuole of Protozoa does not represent the classical Golgi material.

3. The conclusion is reached that the network ('vacuome-Golgi apparatus') in the salivary glands of *Chironomus* larvae described by Parat and Painlevé represents nothing more or less than intracellular canaliculi.

4. The Golgi bodies and mitochondria show no evidence that they are directly associated with the origin of secretory material, such as direct connection with the secretory inclusions or concentration about the intracellular secretory canaliculi.

5. The Golgi bodies, mitochondria, and neutral-red bodies of Parat are discrete in the parietal cells of the salivary glands of the grasshopper.

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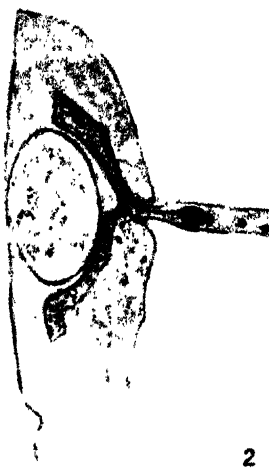
PLATE 1

EXPLANATION OF FIGURES

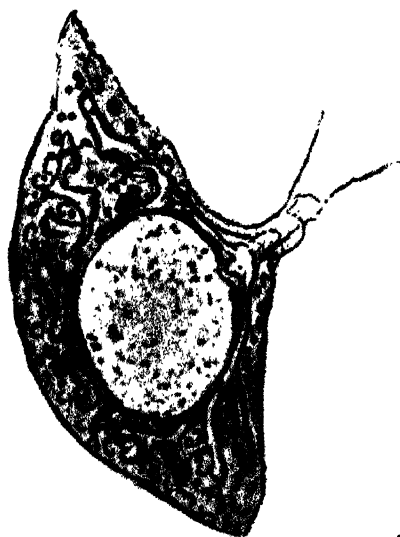
- 1 Parietal cell of salivary gland of *Rhomalea microptera* with complicated intracellular canaliculi. Nasonov technique.
- 2 Parietal cell of a salivary gland of *Melanoplus differentialis* with simple forked intracellular canaliculus. Ludford technique.
- 3 Typical insect Golgi bodies in parietal cell containing intracellular canaliculi. *R. microptera*. Ludford technique.
- 4 Mitochondria and intracellular canaliculi in parietal cell of salivary gland. *R. microptera*. Regaud technique.



1



2



3



4

PLATE 2

EXPLANATION OF FIGURES

All figures represent parietal cells from salivary glands of *Rhomalea microp-
tera* photographed at a magnification of $\times 500$. Nassonov technique.

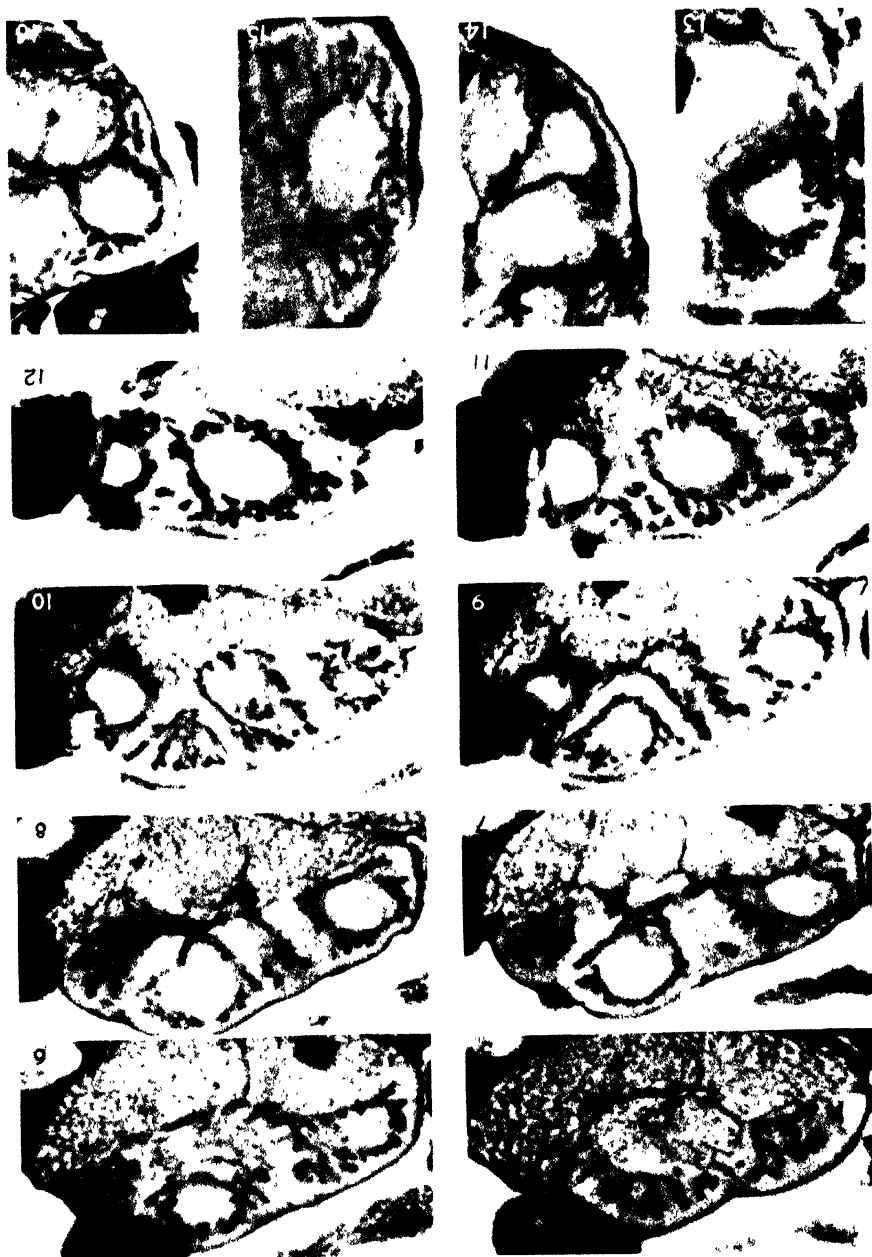
5 to 12 Serial sections of four cells showing branched intracellular canaliculi
with efferent ductules leading to intercellular ducts.

13 Secretory canaliculi joining at apex of cell to form efferent ductule.

14 Efferent ductule leading to intercellular duct.

15 Complex branching of intracellular canaliculi.

16 Another example similar to figure 14.



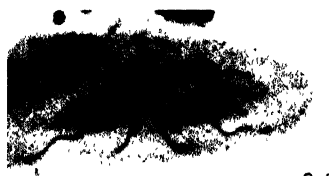
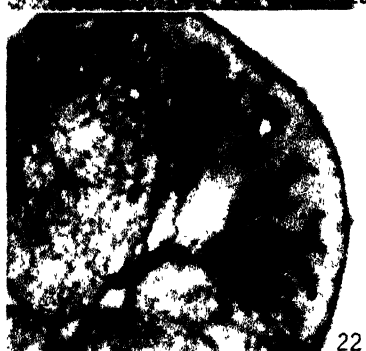
SALIVARY GLAND OF GRASSHOPPER
H. W. HARRIS AND ROBERT L. KING

PLATE 3

EXPLANATION OF FIGURES

17 to 22 Serial sections of a single parietal cell from salivary gland of *Rh-malea microptera*, showing intracellular canaliculi. In many cases the lumina of the canaliculi are clearly shown. The efferent ductule leads directly into intercellular duct. Photomicrographs, $\times 700$. Nassonov technique.

23 and 24 *Paramecium caudatum* with impregnated contractile vacuoles and associated radiating canals. Photomicrographs, $\times 350$. Ludford technique.



23

24

COORDINATION OF CILIARY MOVEMENT

I. METHODS OF STUDY AND THE RELATION OF CILIARY COORDINATION TO CILIARY INHIBITION¹

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TWO TEXT FIGURES AND ONE PLATE (EIGHT FIGURES)

AUTHOR'S ABSTRACT

A simply constructed apparatus, which includes a cinema camera, is described for the recording of ciliary movements. From data for *Modiolus demissus* obtained by this method, it is concluded that the coordinating impulse is a propagated impulse which regulates the temporal and spatial relationships of ciliary contractility, that this impulse has to a certain degree a determinant effect upon ciliary inhibitory influences so that the area rendered inactive is a multiple of the ciliary wave length, that the coordinating impulse is transmissible through cells bearing quiescent cilia, and that it may act as a stimulus of sufficient strength actually to cause quiescent cilia to become active.

Two properties of living substance, conduction and movement, are specialized in ciliated epithelia to the extent that fine thread-like homogenous cellular processes are adapted for rapid contractions and the cells which bear these processes are adapted to conduct impulses which coordinate the rate and time relationships of the ciliary contractions. It is only the occasional observer who has distinguished between these two properties of the ciliated cell. This publication is the first of a series in which certain facts pertaining to the physiological processes producing and regulating ciliary coordination will be presented. The facts obtained thus far support Parker's theory of neuroid transmission in ciliated cells, and for that reason the program of investigation includes a careful comparison of coordination in ciliated epithelia with the known facts of nerve conduction. The underlying principles being the same, the differences in structure make such a comparison of particular value. It remains to

¹The investigation has been made possible through funds granted by the Chemical Foundation and the National Research Council.

be seen whether the cell walls in a ciliated epithelium are comparable to synapses in nerves, how certain drugs which in nerves lower the threshold of conduction across the synapse will affect conduction across ciliated cell walls, and whether any morphological structure comparable to a nerve end organ in a muscle may be demonstrated in ciliated cells.

In order to begin such a program, it is first necessary that a method which is more exact than those previously employed should be developed in order to exclude to a greater extent the personal element in recording data. Also, it must be a measure of the actual movements made by the ciliary waves and not a record of the work accomplished. A review of the literature related to ciliary activity makes it evident that numerous described methods for the estimation of ciliary movement fall into relatively few types, each with its inherent errors and limitations.

DISCUSSION OF METHODS IN USE

Direct observation

The first approach to the study of ciliary movement was, of course, by direct observation. For certain purposes this is a good criterion of ciliary activity, especially when such material as the swimming plates of ctenophores are used. It is still a valuable adjunct to any other method which may be employed. Kuhne ('66), Engelmann ('67, '68), Verworn ('91), Lillie ('01, '08), Parker ('05 a), Williams ('08), Gothlin ('20), Segerdahl ('22 a and b), Gray ('22), Carter ('24), de Rényi ('26), Merton ('27), Nomura ('28), and others have recorded the relative activity in such terms as 'rapid movement,' 'medium amount of activity,' 'slow,' and 'stopped.' Such a method is valuable in that it is based upon a variable of ciliary coordination, namely, the frequency. The tabulated values, however, are subjective estimates without a standard for comparison.

A modified method of direct observation is found in the studies of Bergel ('99) and Seo ('31 b). Ciliated epithelia, when subjected to certain injurious substances in dilute solu-

tion (Bergel, '99), develop 'Flimmerkorpsch' which represent the terminal ends of ciliated cells, which, after separating from the rest of the cell, assume spherical forms. Alverdes ('22) obtained somewhat similar fragments by crushing *Paramoecia*. Umeda ('26) used small clumps of isolated ciliated cells from the frog's mucous membrane. Ciliary activity, locomotion, and the effects of certain drugs on ciliary activity were observed upon these ciliated fragments. The ciliary metachronism was found to be similar to that on the epithelia.

Indirect observation

Propulsion of particles. When the cilia are short and it is difficult to identify separate waves, then it has usually been found expedient to evaluate the degree of ciliary activity by the rate at which overlying particles are propelled, or if the cilia under study happen to be locomotor organs, then their activity is estimated by the rate at which the animal body is carried through the surrounding medium. One aspect of ciliary activity which can more readily be ascertained by the passage of suspended particles than by any other method is the direction of the active phase of ciliary contraction. This is especially true when the cilia are numerous and close together. Sometimes the overlying mucus is sufficient indicator of the direction of movement; usually, however, particles are added which float on the mucous layer or may sink and be propelled directly by the ciliary currents. Carbon particles are satisfactory for studies of ciliary activity in the reproductive and respiratory tracts (Ritter, '59; Heil, '93; Lode, '94; v. Gebhardt, '09; Irving, '24; Hill, '28; and Seo, '31 a). V. Brücke ('17) used India ink. Carmine has been frequently used (Allen, '14; Merton, '23; Irving, '24; Wyman, '25; and Lucas, '32). Fine black sand was found advantageous for the study of direction of movement in molluscs (Kellogg, '15). *Lycopodium* pollen has been used to study the rate of ciliary movement in the trachea (Lommel, '08). *Lycopodium* pollen stained with fuchsin is more easily visible

(Hack, '25). Umeda ('29) employed grains of millet on the ox trachea. A shred of light paper rolled into a small ball was used as an indicator of ciliary velocity by McDonald, Leisure, and Lenneman ('28). Small squares of filter-paper soaked with various ingredients were used by Parker ('05 b) and Parker and Marks ('28) in their studies of reversible ciliary movement as found in the sea-anemone.

Substances such as those already mentioned have been used to determine the degree and rate of ciliary activity, and for this purpose Engelmann ('68) suspended a small sphere by means of a silk thread on the ciliated surface of the frog's mucosa and timed the rate of movement.

When the propulsion of an organism, such as a protozoan, spermatozoan, rotifer, turbellarian worm, snail, or larval form of certain invertebrates, is used, the degree, regulation, and direction of ciliary movement are determined by the behavior of the organism (Jensen, '93; Lillie, '01; Mayer, '11; Copeland, '19 and '22; Alverdes, '23; and Mast and Nadler, '26).

A common method used to measure ciliary activity is the use of a plate-like object which is allowed to rest upon the ciliated surface. Bodwitch ('76) fastened a piece of frog's skin to a solid vehicle and placed this on the ciliated mucosa of the frog. Gray ('24) used a small circular platinum plate.

Certain difficulties and limitations are apparent in the indirect method, whether particles or plates or the movement of the organism is used as a criterion. In the case of particles, differences in size cause differences in rate at which they are transported. The distance separating the floating particles from the vibrating cilia determines how fast the particle will be propelled at any fixed rate of ciliary movement (Gray, '28, p. 82, fig. 65). When the film overlying the cilia is thin, then capillary forces pull the layer in different directions and at different rates in order to maintain an equilibrium. Finely divided particles, such as the various forms of carbon used, remain suspended in the medium and it becomes difficult to differentiate movements due to the cilia from those due to

capillary attraction and gravity. The use of plates overcomes some of the difficulties in that the under surface remains an average fixed distance above the cilia, but with both methods, and also that in which the movement of the entire organism is studied, the thing measured is the flow of the current produced and not the activity of the cilia.

Special devices to determine ciliary activity. Such indirect methods are a measure of the effective work done by the cilia. Wyman (cited from Chamil), Bowditch ('76), and Chamil ('81) in order to measure this work placed a vehicle having a known area upon the cilia and determined the rate of movement when weights of various magnitude were added. The work accomplished by cilia which line tubular structures has been determined by recording the pressure and volume of flow. Parker ('10 and '14) made such measurements on the sponge, Hecht ('16) on the Ascidians, and White ('29) on the Necturus kidney tubule.

Elaborate modifications of the general type, in which a relatively large object rests upon a ciliated surface, have been devised. The first of these is described by Calliburces (cited from Chamil), who used a moist chamber in which the mucous membrane was spread over a layer of cork. A glass cylinder rested upon the ciliated surface and its movements were indicated by a lever attached to the axis. A simple apparatus devised by Grénant (described by Chamil) differs in that the mucous membrane is inclined at about a 45° angle. A cylinder of greater diameter is used so that the surface in contact with the cilia is larger. Ranvier's apparatus (cited from Chamil) has the advantage over the methods already mentioned in that all the cilia of the frog's mucous membrane take part in revolving a glass disc. The disc is submerged in a large vessel of normal saline. Its axis is a fine rod, on the upper end of which is a pointer. The ciliated surface is also submerged and so placed that the cilia will strike against the under surface of the disc near the periphery. More complicated modifications have been constructed; one of these, the 'Flimmermühle,' was reported by Büchner, a student of

Engelmann's. The 'Flimmermühle' was modified in some details by Engelmann ('77), who also constructed the 'Flimmeruhr.' The improvements were in the method of recording the rotation of the cylinder by the cilia. Pith was used for the cylinder instead of glass. The same principle is used in the two forms of the cilioscribe constructed by Dixon and Inchley ('05) and Inchley ('21). In this case the rod is glass and is vertically placed. The mucous membrane is pulled horizontally against the rod. Glass has the advantage over the pith cylinder in that the mucous strings interfere less with the rotation of the cylinder.

When the object of the search is to determine the effect of various drugs, salts, media, gases, electrical stimuli, and stimulation by the nervous system on the mechanism of ciliary movement, then numerous errors are included which are difficult to estimate if a cylinder or disc is employed. The amount of secretion varies under the influence of various reagents, and in experiments where the vascular circulation is maintained, changes in the blood indirectly likewise produce changes in the mucus. The viscosity of secreted mucus is variable, dependent upon the changes in the nature of the secretion and on the rate of evaporation of water from the exposed mucous surface. The secretions tend to clump around the cylinder or disc and hence slow its action. Under these various conditions it is conceivable that the actual beating of the cilia might remain at the same frequency. Chamil has shown that in some cases the cilia may still be active, but fail to register that activity through movement of the overlying cylinder.

Stroboscope. Martins ('84) was the first to use the stroboscope in the study of ciliary movement, and by this method found that the frequency of ciliary contractions was from 10 to 17 per second in the epithelium of the frog's pharynx. These values are higher than those previously made by direct observation. Recently Gray ('30) has employed the stroboscope in the study of ciliary movement of the lamelibranch gill.

Cinema records. Gray's use of motion pictures ('30) has confirmed the direct observations of previous investigators on the form of a cilium during its active and passive phases of contraction. What is still more important, he has calculated from the moving-picture film the time involved in each phase of ciliary contraction. Ciliary coordination and responses to various stimuli can be quantitatively studied only when some such direct and reproducible method of recording the ciliary responses is used. The stroboscope has its limitations in that it cannot be used to record very slow rates of movement; irregular waves and uncoordinated movements cannot be analyzed, and certain subjective difficulties are involved which are fully discussed by Gray ('30). Moving pictures cannot, at least when taken at the rate of 16 per second, record extremely rapid movements. However, the method does show variations within the field and is a permanent record from which a careful analysis may be made.

METHOD EMPLOYED

Moving pictures of cilia on the fresh-water lamellibranch mollusc gill were taken by the author in 1927 and during the summer of 1931 at the Marine Biological Laboratory, Woods Hole. The method was applied to the study of ciliary coordination in *Modiolus*.

Apparatus

With the addition of the moving-picture camera, it was possible to utilize equipment such as is available in most laboratories for this procedure. The adapter to hold the Bell & Howell 70A Filmo camera (figs. 1 and 3) consists of a wooden frame which has three bolts, $10\frac{1}{2}$ cm. long, so placed that one lies at the back of the camera and two in front. Two bars which fit over the bolts are held firmly against the camera with thumbscrews. The adapter is suspended by two right-angle irons, through each of which a bolt is fastened. The camera may thus be adjusted to swing at any angle.

The frame (fig. 1) is composed of two ring stands spaced about 24 inches apart. The rods are about 3 feet high. With right-angle clamps a horizontal bar is fastened near the upper ends of the ring stands, and from it are suspended the adapter and camera. The ring stands are made rigid by extending rods from them to any solid object upon the wall to which they may be securely fastened. The bases of the ring stands are fastened firmly to the table top with clamps. The whole is quite rigid and moving pictures taken of a stage micrometer show no irregularities due to the vibration of the camera.

A bellows of some sort is necessary. A set of brass tubes of various lengths were made which have an inside diameter large enough to pass over the eyepiece of the microscope. Threads on the opposite end are of such size that they will screw into the opening occupied by the lens. The entire lens system is removed. The brass tube is lined with black velvet.

For some work a focuser which shows the size and position of the object at the film level is employed, while in other cases it is not required. The one recommended by Mitchell ('30) is the Goertz model A and has proved satisfactory. When the reflex focuser is used, it is inserted between the brass tube and the camera. For focusing in the microscope, a demonstrating ocular was satisfactory and replaced the microphote described by Mitchell.

Cinema film

A supersensitive panchromatic 16-mm. film was used. It is usually necessary to know the rate at which the frames of film are passing across the camera opening. Once this is determined, the error under various conditions and at various times is smaller in many cases than the experimental error involved in measurements of the records. Gray ('30) has described a device for recording the exact time intervals directly on the film. If the camera possesses a winding key, a cogged wheel with an axis made to fit snugly in the winding-key socket may be used, and its rate of rotation recorded simultaneously with some available timing device. This

method, however, requires a tedious calibration of the film after it is developed.

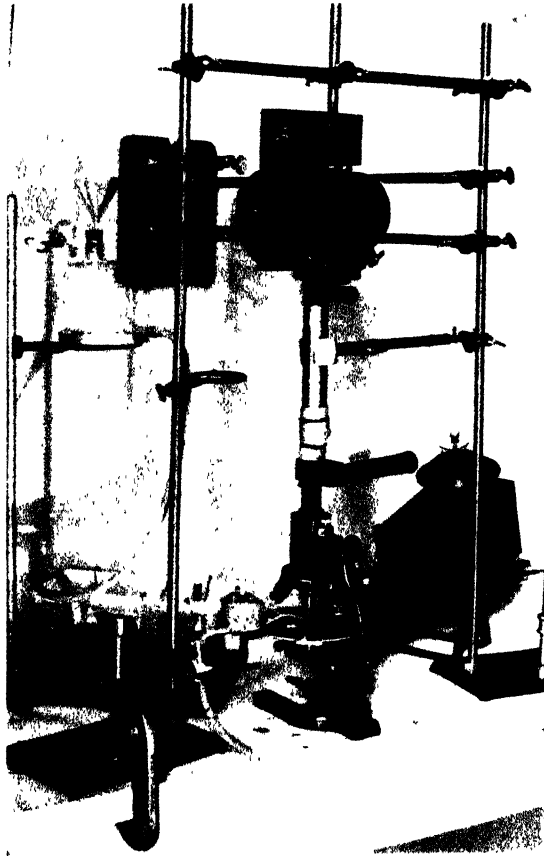


Fig 1 A photograph of the apparatus used to obtain cinema records of ciliary movement at a magnification of about 136 times.

Chamber

Since ciliated epithelia are sensitive to slight changes in the surrounding medium, it is necessary that they be examined under constant conditions in which the waste products

are constantly removed. The chamber illustrated in figure 4 permits the examination of cilia in a continuously flowing stream of liquid. The walls of the chamber are made of glass tubing, the end of each bent to form two sides of the square and then fused into one piece. One end of each of the original glass tubes is left open, the opposite end is closed. In the side of each tube which opens into the chamber a small hole is blown through which the circulating medium enters and leaves the chamber. The top of the chamber is a cover-slip (fig. 5). It is first prepared by rimming the edge of the glass with a melted mixture of beeswax and rosin. A lamella of the mollusc gill or other ciliated epithelium is placed on the center of the cover-slip with a small amount of water. Finely drawn glass rods are placed across the ends of the preparation, and the ends of the rods held against the slip by pressing them into the rim of wax with a warm knife. The preparation is inverted over the chamber and the cover-slip is sealed to the chamber when melted wax is added to the crevices between the chamber and cover. If the mixture of wax and rosin is correct, the fluid may be circulated almost immediately through the chamber without disrupting the walls. The preparation so held against the cover-slip permits the use of high magnification, and the beating of the cilia does not carry the epithelium across the field.

Illumination

The source of illumination may be either an arc lamp or bright filament lamp. A 500-watt baloptical bulb in a microscope lamp was found to be satisfactory, and permitted pictures to be taken with the microscope diaphragm closed to about 1 mm.

OBSERVATIONS AND DISCUSSION²

The results obtained with this apparatus are shown in figures 6 to 10. With a permanent record of ciliary movement in hand, a closer analysis of ciliary activity may be

² The author wishes to acknowledge his appreciation of the many helpful suggestions and discussions related to this subject by Dr. G. H. Bishop, of Washington University.

made than is possible with the eye alone, and this method of direct estimation of ciliary activity eliminates the errors which are likely to be involved in recording the rate at which particles are carried by an overlying fluid layer produced by ciliary vibration.

The movements of cirrus-like laterofrontal cilia may be traced in figure 6. It has not been determined in the case of these cilia in which direction the coordinating impulse passes through the epithelium, for the reason that the cilia on alternate cells are in approximately the same phase of contraction. This is clearly illustrated in the right side of the cinema frames of this figure. A surface view of lateral ciliary waves somewhat out of focus is shown below the laterofrontal cilia. Their movement is toward the left of the print.

A profile view of the lateral ciliary waves is shown in figure 7. Several hundred cilia are involved in the formation of one ciliary wave. The active phase is indistinct. The crest of the larger passive wave is used in making measurements of wave length and velocity. A single wave as it moves toward the left is identified by a cross. The temperature of the sea-water circulating through the chamber was 15°C . One millimeter on the film is equal to $7.3\ \mu$. The pictures were taken at the rate of 16 frames per second. The velocity of wave movement is therefore $49\ \mu$ per second, the wave length $15.6\ \mu$, and the frequency 3.1 vibrations per second.

Figures 8 and 10, a to g, are surface views of lateral ciliary waves and figure 9 is a profile view. The temperatures of the preparations used are: figure 8, 11°C .; figure 9, about 25°C .; and figure 10, 9°C . The location of the wave crests in figure 10 are measured and plotted in figure 2. The abscissa is the distance from left to right of the microscopic field shown on the film. The ordinate is the cinema frames, and prints of these frames from the third to the sixty-fifth are shown in figure 10. Each dot locates the crest of a ciliary wave. In figure 10 the velocity of movement is approximately $37\ \mu$ per second, the wave length averages $10.3\ \mu$, and the frequency is 3.6 per second.

Parker ('05 a) interpreted evidence secured from his study of the swimming plates of ctenophores that ciliary movement is coordinated by the transmission of nerve-like or neuroid impulses through the ciliated epithelium. Additional evidence supporting Parker's theory will be presented in the second paper of this series, in which it will be shown that the conductile mechanism of ciliated cells and nerves have certain properties in common.

The vibrating lateral cilia frequently cease all movements. The cessation of activity is not due to changes in the external fluid environment, such as increased acidity, lack of oxygen, accumulation of carbon dioxide, or presence of waste products, for the reasons that cilia on only a portion of a filament may become inactive, and that under the same external conditions they may become active again. Cessation of activity under these conditions does not make itself apparent by degrees of decreasing activity, nor does it necessarily occur when the ciliary activity, in general, is sluggish. Cessation of movement is sudden and complete. This is evident in figure 9, in which two of the waves present in the third frame disappear by the time the fourth frame is taken. It may involve a short distance, as shown in figure 9, or it may be several millimeters long or more. It may last for only a second or even less, or it may last for several minutes. Renewed activity at the end of the quiescent period is as vigorous or even more so than before. A similar phenomenon has been described by Carter ('26) in the cilia used for locomotion of the nudibranch veliger. His investigations indicate that in this animal spontaneous stoppages of ciliary activity are produced by nervous stimuli. Such a stimulus may be due to an inhibition impulse. The terms inhibition and inhibition impulse have been used in the present study of the lateral cilia of the mussel gill for the reason that the cessation of ciliary movement is complete and sudden and is not preceded by any discernible change. The phenomenon differs in the two animals in that the inhibition impulse occurs in the veliger at regular intervals, whereas in the

Mytilidae it may not appear for hours at a time or it may occur numerous times in relatively rapid succession. The term impulse has been applied because its visible effect on the cilia suggests that some influence originating outside the ciliated cells is acting upon them. Its origin in the case of the Mytilidae gill is not nervous because nerve fibers are apparently lacking (Lucas, '31) in those regions of the filaments which bear the cilia.

Although the origin of the inhibition impulse may be in doubt, its relationship to the coordination impulse is clearly demonstrated by means of the cinema film records. In most cases it is found that the line at which the approaching ciliary waves terminate remains fixed and does not encroach upon the quiescent cilia. The waves which begin on the opposite side of a quiescent area likewise begin from a line which remains fixed. Such an example is shown in figure 8. An exception to this is shown in certain parts of figure 10, a to g, in which there is a rhythmic movement of this line back and forth. Its significance will be discussed later. The waves which begin or terminate at an inhibited region have the same period, wave length, and velocity as those farther distant from such an area. Evidence of acceleration or retardation of movement has not been discovered.

It has already been stated that sometimes the length of the region under the influence of the inhibition impulse is short, as is shown in figures 9 and 10, and measurements made of its length reveal the fact that it is equal to or is a multiple of the length of one ciliary wave. In figure 10, f and g, the inhibited area is equal to one wave length. In figure 9 it is equal to two waves. A region of three waves inhibited simultaneously has not been recorded, but it is thought to have been observed. It would seem from these data that the coordinating impulse which is the determining influence in the spatial and temporal relationships of ciliary contractions is also effective in integrating the action of the inhibiting impulse, so that coordination on the two sides of the inhibited area is not disrupted.

Evidence that coordination is maintained on both sides of an inhibited area is presented in figure 10, a to g, and its graphical representation in figure 2. The waves that appear at the left side of the film are separated by a quiescent area

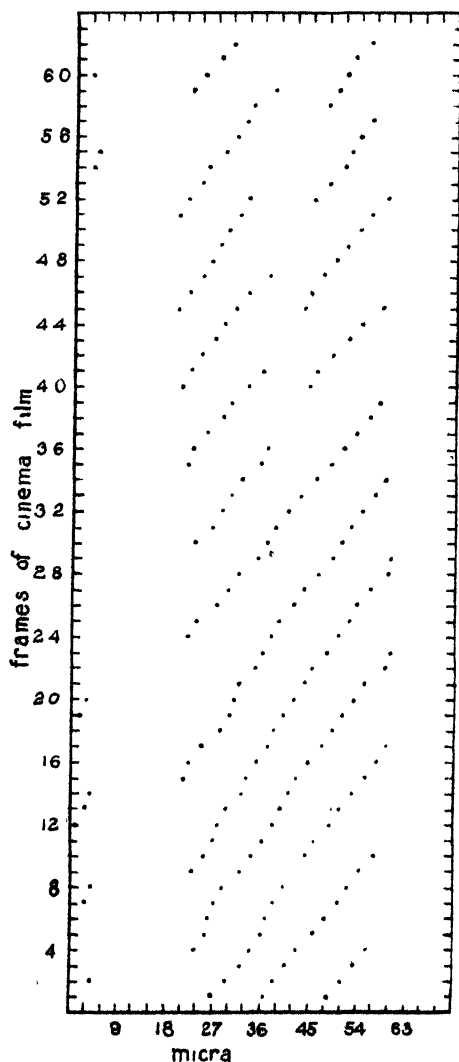


Fig. 2 A graphical representation of the moving ciliary waves obtained from records given in figure 10. The crest of each ciliary wave on each frame of the cinema film is located with a dot.

equal to one wave from the ciliary waves in the center of the film. Lines projected from the rows of dots on the left side of figure 2 coincide with the row of dots which begins to the right seven to nine frames later. The continuation of the projected lines across a quiescent area is more strikingly shown in the upper part of figure 2. This means that the waves which begin on one side of a quiescent area are coordinated in their movements with those that terminate on the opposite side.

The question which arises at this point is whether or not coordination is continued on both sides of a quiescent area due to the fact that the old rhythm is maintained after the inhibition impulse knocks out one, two, or three complete waves, or whether the coordinating impulse is transmitted from the region of terminating activity on one side through the quiescent area and causes the region on the opposite side to begin its activity in its corresponding rhythmic relationships. The latter interpretation is more probable, as will be shown from data obtained in figure 10.

Behavior of the waves recorded in figure 10 reveals a variation from the previously described normal behavior of the quiescent cilia in that the limits of the area do not remain in a fixed place, but instead oscillate back and forth. The length of the oscillation at the right end is equal to one wave length and presumably it is the same at the left end, which, however, is recorded only in part on the film. This is brought about by the fact that the quiescent cilia at the two ends of the inhibited area become periodically active. The wave which appears at the left of frame 6, for example, is being formed by cilia which a few frames previously were inactive. Its movement to the right indicates that the propagation wave and hence the coordinating impulse is in that direction. At a more or less definite place, as shown in figures 10 and 2, the wave terminates.

A new wave on the right side of the quiescent area does not arise as it does in figure 8, namely, from a fixed unchanging line, but is formed by previously inactive cilia and as it

moves to the right, the cilia in its train again become inactive.³ When the wave has progressed to the right a distance equal to one ciliary wave length, a second wave begins out of the same inactive cilia from which the first one started. Thus the new wave arises in perfect coordination with the one which is moving in front of it. Since this is so, it seems reasonable to assume that it is the coordinating impulse which is responsible for initiating activity in quiescent cilia, in their proper spatial relationship to the surrounding vibratile elements.

Such an impulse must arise in this case either from the activity of the wave which has moved to the right or else from the wave which terminated at the quiescent area. In the latter case the impulse must then have passed through a region in which the cilia were inactive. The latter suggestion is the more tenable, in view of the fact that these ciliated cells have an irreversible physiological polarity which determines that the ciliary waves on any one epithelial row shall always move in the same direction. Moreover, it is hardly to be supposed that the coordinating impulse would move in a direction which would be opposite that of the propagation wave, but rather it is most probable that these moving ciliary waves are the visible expression of the coordinating impulse.

CONCLUSION AND SUMMARY

Methods previously used to study ciliary activity are reviewed and certain errors discussed.

A simple apparatus for recording ciliary activity is described; also a chamber in which the fluid environment of the ciliated epithelium may be maintained under constant conditions.

From the records obtained on the cinema film it is concluded that the coordinating impulse is a propagated impulse

³ A certain refractile body on the filament is somewhat confusing because it resembles a ciliary wave. It is easily differentiated, however, from the waves in the projected moving film. The arrow to the left in frame 13 of figure 10, b, indicates this refractile body and the arrow on the right indicates the wave. In frame 12 the two are almost superimposed.

which regulates the temporal and spatial relationships of ciliary contractility, that this impulse has to a certain degree a determinant effect upon ciliary inhibitory influences so that the area rendered inactive is a multiple of the ciliary wave length, that the coordinating impulse is transmissible through cells bearing quiescent cilia, and that it may act as a stimulus of sufficient strength actually to cause quiescent cilia to become active.

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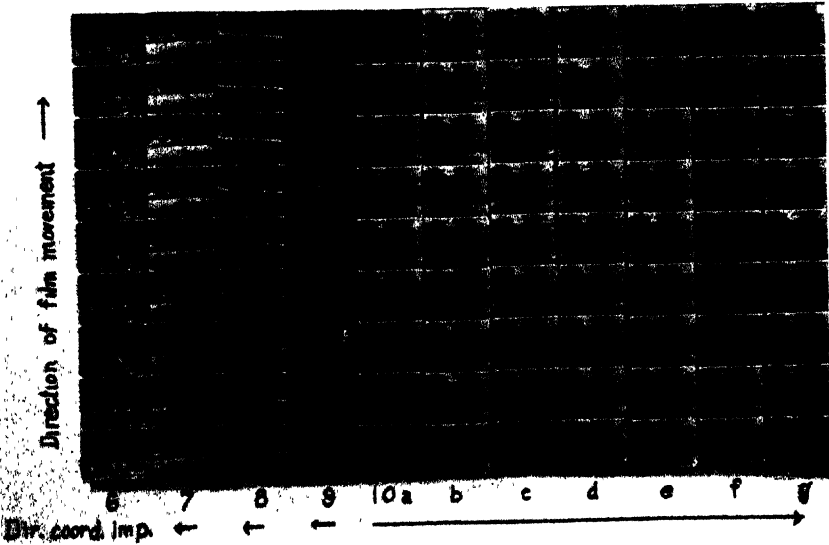
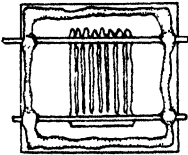
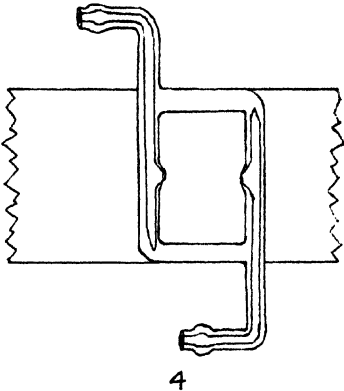
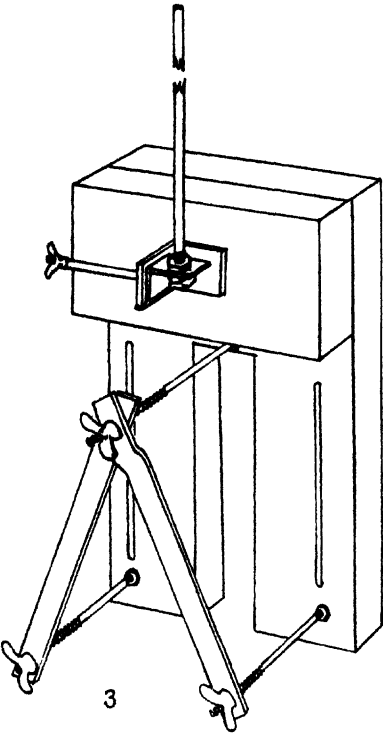
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PLATE 1

EXPLANATION OF FIGURES

- 3 Adapter designed to hold a Bell & Howell Film 70A camera.
- 4 Chamber through which a fluid medium may be circulated in order to maintain a preparation of ciliated epithelium under constant conditions.
- 5 Cover-slip for the chamber showing arrangement of the ciliated preparation which in this case is a section of *Modiolus* gill.
- 6 Strip of film recording the vibrations of laterofrontal cilia. One millimeter on the film in figures 6, 7, 8, and 10 is equal to $7.3\ \mu$.
- 7 Profile view of lateral cilia. *Dir.coord.imp.*, direction of the coordinating impulse, is toward the left in figures 7, 8, and 9 and toward the right in figure 10. Crosses identify one of the waves in its movement along the filament.
- 8 Surface view of lateral cilia. The junction between the inactive cilia to the right and the active cilia to the left remained fixed during the period the records were taken.
- 9 Inhibition of two ciliary waves. One millimeter on the film equals $8\ \mu$ on the preparation. The velocity of wave movement to the left is $138\ \mu$ per second, the average wave length is $16\ \mu$, and the frequency is 8.5 vibrations per second.
- 10, a to g Surface view of lateral cilia in which certain relationships between the coordinating and inhibitory impulses are recorded.



COORDINATION OF CILIARY MOVEMENT

II. THE EFFECT OF TEMPERATURE UPON THE CILIARY WAVE LENGTH¹

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TWO FIGURES

AUTHOR'S ABSTRACT

The ciliary wave length is a constant in the temperature range of 10° to 30°C. and in *Modiolus demissus* has the average value of 13.1 μ . Comparison of these data for ciliary coordination with those of nerve conduction probably indicates that the two physiological processes possess certain properties in common. The cells which bear the cilia are 12.75 μ long and the variation in ciliary wave length and cell length is about the same, which may indicate a possible cytophysiological relationship between the two.

Both mechanical and physiological explanations of ciliary coordination have been advanced. Verworn ('91) postulated that a metachronous rhythm was set up by the striking of one cilium against the next. Upon this assumption is based the idea that coordination is the result of purely mechanical influences. Parker ('05) has cited examples in which the wave movement progresses in a direction different from that of the effective beat of the individual cilia, and suggests that a primitive nerve-like or neuroid impulse is transmitted along the ciliated epithelium. Evidence for the existence of a primitive type of nerve-like conduction is found in the works of Engelmann ('68), Kraft ('90), Parker ('05, '19), Lillie ('14), Wyman ('25), and others. It is derived for the most part from the fact that the reaction of cilia to stimuli is transmitted to other cilia across a region of epithelium upon which the cilia have been rendered inactive. Since the effects of greater activity in one part of an epithelium are transmitted to another part, it is assumed that coordination, itself, is governed by the same conductile mechanism. Somewhat

¹The investigation has been made possible through funds granted by the Chemical Foundation and the National Research Council.

more direct proof that this is the case has been presented in part I of this series from records obtained on cinema films of ciliary coordination and ciliary inhibition (Lucas, '32). It was concluded as a result of this study that the coordination impulse regulates the temporal and spatial relationships of ciliary contractility, that this impulse has to a certain degree a determining effect upon ciliary inhibitory influences, so that the area rendered inactive is a multiple of the ciliary wave length, that the coordinating impulse is transmissible through cells bearing quiescent cilia and that it may act as a stimulus of sufficient strength to cause quiescent cilia to become active. Additional evidence that the ciliary coordinating impulse is nerve-like in its physiological properties is presented in this paper.

I wish to express my appreciation to Dr. G. H. Parker and to Dr. G. H. Bishop for criticisms and suggestions related to this work. The study was carried on at the Marine Biological Laboratory, Woods Hole, Massachusetts, and at Washington University.

MATERIAL AND METHODS

The lateral cilia on the filaments of the outer gill lamellae of *Modiolus demissus* were used. The apparatus employed to obtain cinema records of ciliary movement has been described (Lucas, '32). Observations of the preparation were made by using a closed chamber of approximately 0.7-cc. capacity, and through which flowed normal sea-water at the rate of about 15 cc. per minute. The gill filaments were held against the glass cover of the chamber by means of fine rods which were sealed in place.

The temperature to which the cilia were subjected was determined by averaging the temperatures of the water before it entered and after it left the chamber. An error of one-half degree in the average was permitted.

A half hour was allowed to elapse after the preparation was set up, before any records were made. From then on at

intervals of about ten minutes strips of film 1 to 2 feet in length were exposed. The nature of the records is shown in the first paper of this series. From one to ten records were obtained from each individual used; each preparation was subjected to only one temperature. The number of animals used and the number of records taken equalize individual differences.

Records were made only of waves which moved regularly across the field. Irregularities such as those described by Gray ('30), in which successive waves of a row are increasingly far apart, were omitted.

Measurements of cell size were made with an eyepiece screw micrometer upon living, unstained tissue in which the cilia had ceased to beat due to the accumulation of waste products such as occur in the ordinary microscopic preparation.

OBSERVATIONS

Much of our knowledge of physiological processes related to nerves has been gained by a close analysis of the velocity of conduction, the period, and the form of the electrical wave which accompanies stimulation. Thus far, similar exact analyses have not been attempted as a means of determining physiological processes which underlie ciliary coordination. The variables of wave movement are the same in nerves and in ciliary waves, namely, frequency or period, wave length, and velocity. It is obvious that data concerning these variables for ciliary movement would readily lend themselves to comparison with our more extensive knowledge of nerve conduction. It is known that frequency (number of ciliary vibrations per second) and the velocity (the rate at which the propagation impulse is traveling) varies under different conditions, because such changes are evident in the speed at which overlying particles or objects are propelled along the surface, but it is not known whether these two variables increase proportionally. Both Gray ('30) and Lucas ('31) have independently suggested that the two variables do tend to remain in the same proportionate relationship, with the

result that the third variable tends to be a constant, but neither author has presented sufficient data to substantiate this conclusion.

In table 1 is summarized the results of 241 records of wave length taken at temperatures which ranged from 10° to 30°C. The variation in wave length at each temperature is approximately 10 μ and the limits in this variation are about the same at each temperature. It has already been pointed out that the records were taken where the waves were moving smoothly and regularly. In cases where the waves are irregular the wave length may be greater or less than the limits given in table 1. The average wave length at each temperature is

TABLE 1
Ciliary wave length

TEMPERATURE, CENTIGRADE	NUMBER OF DETERMINATIONS	VARIATIONS IN WAVE LENGTH	AVERAGE WAVE LENGTH
		<i>Micra</i>	<i>Micra</i>
10°	43	8.9-19.8	13.1
15°	66	10.1-20.8	13.0
20°	49	9.9-18.4	12.7
25°	56	10.8-17.7	13.6
30°	27	9.3-19.0	13.1
Total, 241		Final average, 13.1 μ	

approximately the same. The final average has value of 13.1 μ .

The question arises whether the wave length is determined by any physical part of the ciliary mechanism. In the effort to determine this point a careful study of the form of the lateral epithelium and its component cells was made, as well as the relation of the ciliary waves to this epithelium.

The lateral epithelium is four cells wide and extends the length of the gill filament, as described by Engelmann ('80) and as shown in figure 1. The epithelium lies close to the frontal edge of the filament. Each cell row (*C.R.*) has been designated for convenience with a number beginning with the row toward the frontal edge. Cell rows 1, 2, and 4 are about equally wide, 4.4 μ , 4.6 μ , and 4.0 μ , respectively. The

third row is narrow, 1.5μ . Measurements of the total width average 14.5μ , as given in table 2.

The variation in width from 12.5μ to 16.8μ is relatively small. The regularity of the width of each row of cells and the row as a whole stands in marked contrast to the irregularities present in the lengths of the cells.

Measurements of cell length were made mostly upon cell rows 1 and 2 because the transverse wall could be distin-

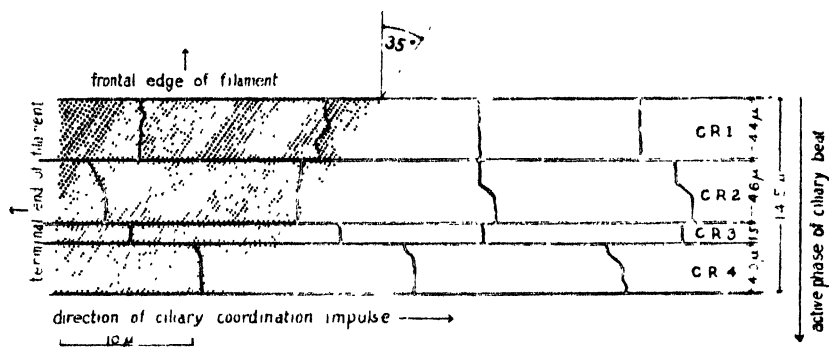


Fig. 1 Surface view of the lateral ciliated epithelium of *Modiolus* gill. The dots represent basal bodies as seen in surface view, which are distributed over the surfaces of all the cells.

TABLE 2		
<i>Width of four lateral cells</i>		
<i>Number of measurements</i>	<i>Variation in width</i>	<i>Average width</i>
20	12.5μ – 16.8μ	14.5μ

guished with less chance of error. The values recorded are presented in table 3. The average cell length is about the same for the first two rows and enough measurements were made upon rows 3 and 4 to indicate that they fall within a similar range of variation. The average cell length is 12.75μ ; the average ciliary wave length, 13.1μ , is only 0.35μ greater. Statistically it is doubtful whether this difference is significant. The close similarity in the length of the cell and the length of the ciliary wave may indicate a cytophysiological relationship between the two. Moreover, the distribution of

variation in cell length and ciliary wave length is about the same, as is shown in figure 2. Their respective values are distributed to form two unimodal curves whose arithmetic

TABLE 3
Cell length

CELL ROW	NUMBER OF MEASUREMENTS	VARIATION IN CELL LENGTH	AVERAGE CELL LENGTH
		<i>Micra</i>	<i>Micra</i>
1	80	9.8-15.8	12.7
2	59	9.5-18.5	12.8
Total, 139		Final average, 12.75 μ	

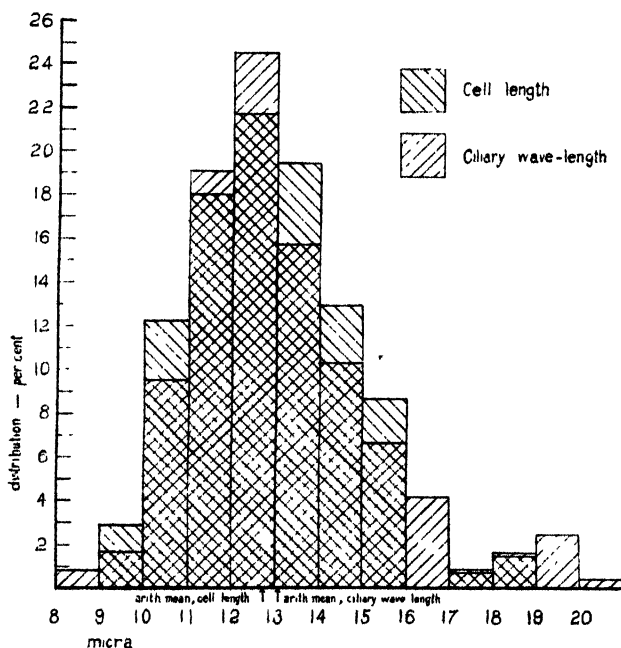


Fig. 2 The distribution of variability in ciliary wave length and cell length are superimposed in this graph.

means approximately coincide. The irregular distribution of the values above 18 μ along the abscissa is probably due to the small numbers recorded for this region of the variation range. Were the total count for cell length and ciliary wave length more numerous, it is probable that such irregularities

would be absorbed in the right slope of the curve and the two arithmetic means would be even nearer the same value than they are now.

Examination of individual transverse cell walls (fig. 1) makes it clear that they lack the regularity of contour which is characteristic of the longitudinal walls. The transverse boundary of a cell in any one row may or may not be in line with a similar boundary in an adjacent row. There is not the regularity depicted by Engelmann in his diagram of this epithelium.

The arrangement of the basal bodies are clearly shown in surface view under high magnification. Engelmann ('80) reported that the basal bodies on the lateral cells of certain molluscs were arranged in rows orientated obliquely at a 45° angle across the surface of the cell. In *Modiolus demissus*

TABLE 4		
<i>Angle of basal body rows</i>		
<i>Number of measurements</i>	<i>Variation in angle</i>	<i>Average angle</i>
20	23° – 39°	35°

(table 4 and fig. 1) the angle of inclination averages 35° with a relatively small variation, part of which is probably due to personal error in making measurements. The rows of basal bodies on one cell seem to be so aligned that they are continuous with those of adjacent cells. The basal bodies extend to the margin of the cell surface and the cell boundaries do not disrupt the regularity of their arrangement.

It was thought that the arrangement of the basal bodies in diagonal rows might have some relationship to the angle of the wave front of the moving ciliary wave. This does not seem to be the case, however, because the ciliary wave front is usually at right angles to the long axis of the lateral epithelial row. Sometimes there occurs a slight inclination in the angle of the wave front in the direction of the propagation wave, but it is only about 10° and never assumes an inclination as great as that of the basal body rows.

Each cilium vibrates transversely across the epithelium so that its effective beat is away from the frontal edge of the filament. The propagated wave which is set up moves at right angles to the plane of ciliary contractions. Since the wave front is in the same plane as the ciliary contractions, it is evident that all the cilia along a transverse line across the epithelium are beating together synchronously. These lines of synchronous movements set up a metachronous rhythm along the axis of the epithelium to produce the visible appearance of a propagated wave. This wave never changes its direction of progression.

DISCUSSION

It is evident from the facts presented that the frequency of ciliary vibration and the velocity of the propagation wave vary proportionately on the average. The average wave length is, therefore, a constant, having a value of $13.1\ \mu$. Comparison of these facts with those related to nerve conduction indicate a close similarity in the fundamental physiological processes which are operating in both cases. The exact measurement of wave length in the action current of nerves is difficult to determine directly. Most records are secured from the rate at which the current develops, the velocity of its transmission, and the amplitude of the current. Lillie ('14, '23) points out that in general the rate at which the current rises to the maximum is proportional to the velocity of transmission and it is interpreted that the intensity or amplitude of the current which increases with increasing temperatures remains proportional to the velocity of maximum rise in the action current. This results in a tendency for the wave length to remain constant. The relationships of variables involved in nerve activity at different temperatures have been reported by Gasser ('31). In one instance the record presented graphically (lower half of fig. 3 in Gasser's publication) shows that curves for spike duration and conduction time are parallel, which means that the length of the wave is constant. In the set of values which Gasser

presents in the upper half of figure 3, the two curves mentioned above diverge slightly as the temperature is lowered. Inspection of the variation in wave length of table 1 makes it evident, in the case of cilia also, that a similar variation in the wave length may occur. These variations are to be found not only in different individuals, but at different times in the same individual and the same group of ciliated cells.

It is evident that the relationship of the variables involved in ciliary wave movement and in the nerve action potential tend to remain the same at different temperatures. Not only are the two alike in this respect, but an additional similarity was found in the behavior of each to temperatures below 10°C. Gasser noted in nerves a marked prolongation of physiological processes at low temperatures. Coordination of ciliary movement in the lateral cells was so disrupted and irregular at temperatures less than 10° that no records were obtained. The mollusc employed was studied during the summer months.

On the basis of these comparisons between nerves and coordinated movements of cilia, and from the evidence presented in an earlier paper (Lucas, '32), it is concluded that the cilia are coordinated by the transmission of an impulse through the epithelium and that this impulse is nerve-like in its physiological properties.

An epithelium differs from a nerve fiber in that cell walls are numerous along the conduction pathway. Their effect upon the velocity of conduction is stated by Lillie ('14, p. 430), "the presence of cross-partitions at intervals would decrease the electrical conductivity of the tissue in the longitudinal direction and thus lessen the distance along which the local electrical effect would be transmitted with an intensity sufficient for excitation." That this might hold for ciliated cells also is indicated by the fact that the conduction rate is less in the laterofrontal epithelium where the transverse boundaries of cells are about five times as numerous as they are in the lateral epithelium (Lucas, '31).

It was found that the average ciliary wave length was only slightly greater than the average cell length. Can the cell

be regarded as a structural unit which limits the extent of cyclic physiological changes concerned with conduction? Evidence that this might be the case is derived from the fact that the coordination impulse may arise in any ciliated cell of an epithelial row when the physical continuity with the preceding cell is broken, and that an isolated ciliated cell, as described by Bergel ('99), shows coordinated metachronous movements. On the other hand, the preponderance of evidence indicates that the coordination impulse passes through an epithelium without reference to cell boundaries. There is no visible irregularity in the wave when it is crossing a cell wall; the wave front is a straight line which is not changed by the irregularities in the transverse arrangement of the cell boundaries; and finally the ciliary waves which are passing across any one group of ciliated cells may at different times vary in length from less than 9μ to more than 20μ . Therefore it is concluded that the length of the cell surface does not directly determine the length of the ciliary wave.

Since interpretation of the facts leads one to the conclusion that the contractile and the coordinating mechanism are distinct physiological functions of the ciliated cell, then each should be able under certain circumstances to act independently of the other. Evidence that this may happen is available. Laterofrontal and lateral cilia have been observed to beat synchronously. In one case the cilia on twelve laterofrontal cells beat in such a manner. The cells had not been separated from the epithelium and the cilia in neighboring regions vibrated metachronously. A synchronous rhythm is the type which is set up when mechanical factors alone are acting. Therefore, when synchronous movements occur the coordinating impulse has ceased to be effective. That the coordination impulse may act independently of ciliary movement has been shown by Lucas ('32), who found that the metachronous rhythm is maintained across an interval in which the cilia are quiescent.

Each ciliated cell is, therefore, a nerve-muscle-like complex and somewhere between the two there probably exists a neuro-

muscular type of junction. The basal body which is connected with the end of the cilium, and which is in contact with the cell surface, may prove to be the locus of this junction.

SUMMARY

1. The ciliary wave length of the lateral epithelium in Modiolus gill is constant at temperatures from 10° to 30°C. and has an average length of 13.1 μ .

2. Comparison of the visible phenomena related to coordination with the present knowledge of related phenomena in nerves indicates that coordination is due to the passage of a relatively slow-moving impulse transmitted through the non-vibratile parts of the ciliated cells.

3. The average length of the lateral ciliated epithelial cells is approximately the same as the ciliary wave length, but thus far evidence is lacking that this dimension of the cell directly determines the extent of protoplasm which shall be involved in the cyclic change associated with the production and transmission of a coordination impulse.

4. Since contractions of the cilia and the transmission of the coordination impulse may occur independently, it is concluded that there exists a neuromuscular type of junction across which the impulse passes when the two are functioning in unison. It is suggested that the basal body may be the locus of this neuromuscular connection.

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A TOPOGRAPHICAL AND CYTOLOGICAL STUDY OF THE SYMPATHETIC NERVOUS COMPONENTS OF THE SUPRARENAL OF THE CHICK EMBRYO¹

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FIVE TEXT FIGURES AND ONE PLATE (THIRTEEN FIGURES)

AUTHOR'S ABSTRACT

A study was made of the development of the suprarenal gland of the chick with reference to the following points: 1) Topographical relationship of the sympathetic cell masses to the cortical cords, and to the sympathetic nervous system. 2) Cytological differentiation of the sympathetic cells.

The migrating indifferent sympathetic cells, although becoming generally distributed over the medial surface of the cortical ridge, form a series of more prominent aggregations at certain definite loci. These aggregations are the rudiments of two longitudinal ganglionic chains, which develop from the sixth to the seventh day. Around and between the ganglionic rudiments the indifferent cell groups become penetrated by sympathetic fibers from the sympathetic-chromaffine complex. At the same time multipolar sympathetic cells lying between the cortical cords send processes to the tracts developing between the ganglia on the surface of the cortical ridge.

Differentiation of 'chromaffine cells' from indifferent cells of the aggregations begins during the eighth day, after a profuse innervation of the rudiments has been established. Differentiation is evidenced by the appearance of cytoplasmic granules, and by the appearance of the 'chromaffine reaction.' The possible bearing of the innervation of the indifferent cell aggregations upon their differentiation and upon their penetration of the cortical bodies is discussed.

INTRODUCTION

From the extensive literature on the development of the suprarenal gland (Poll, '06), the points of agreement of most of the present investigators are: the gland has a double origin, the cortical portion is derived from a cellular proliferation of the coelomic epithelium, and the medullary portion from cells of the sympathetic nervous system. The latter cells arrive in ultimate relationship to the cortical portion by an active penetration beginning some time after the two elements first come into contact with each other.

¹ This work was done largely at the zoological laboratory of the University of Kentucky during 1929 and 1930. It was completed during the author's leave of absence from the University of Kentucky, while in residence at The University of Chicago, 1930 and 1931.

The suprarenal of the chick, with which this paper deals, as that of birds in general, differs from the suprarenal of mammals in that the cortical tissue and the medullary tissue are topographically not arranged as the names *cortex* and *medulla* imply, but are irregularly intermingled in the form of cords. Again, the development of the chick's suprarenal is complicated in that the sympathetic system, from which the medullary tissue takes origin, arises in successive steps. These rather definite stages are called the primary and the secondary sympathetic chains, respectively (His, '97). Rau and Johnson ('23) have shown that the so-called primary chain is but a transitory stage in the development of the true sympathetic chain, and that the elements of the former are incorporated more or less completely into the latter.

The so-called primary chain is by a number of authors considered to be responsible for the contribution of the medullary primordium as well as for the innervation of the glands (Rabl, '91; Fusari, '93; Soulie, '03; Rau and Johnson, '23). Of these, Rabl holds that the migration of elements to the cortical rudiments also continues from the 'secondary sympathetic chain.' Abel ('12) describes a migration from the sympathetic system as late as the seventh day, but, according to her, these cells differentiate into sympathetic nerve cells in this region. Goormaghtigh ('21) has likewise seen a migration of sympathetic cells from the primary sympathetic chain, but, according to his view at that time, the sympathetic cells are intermingled with mesodermal cells which give rise to the medullary tissue, while the former contribute the nervous elements of the suprarenals.

Recently, the problem of the formation of the suprarenal glands in the chick was analyzed experimentally. Willier ('30), by means of the chorio-allantoic grafting method, demonstrated that histologically complete glands could be obtained from isolations of the wolffian body, containing the suprarenal rudiment, made as early as ninety-six hours. He thereby showed that the cells contributed by the primary sympathetic chain are sufficient for the formation of the

medullary cords. The work further suggested that the cells derived from the primary sympathetic chain form a contiguous mass on the surface of the cortical cords, and penetrate between the latter after lying in juxtaposition to them for several days.

In the above-mentioned work, of which this paper is a direct outgrowth, a number of questions were raised relative to the significance of the relationship between the sympathetic system and the suprarenal, and to the differentiation of the medullary tissue of the gland. It is the purpose of the present paper to record the results of a study of the following points: First, the topographical relationships between the sympathetic cell groups and the cortical cords established prior to the differentiation of the medullary tissue. Secondly, the relationships at the time of cellular differentiation between the sympathetic cell groups and the cortical cords on the one hand and the sympathetic cell groups and the sympathetic nervous system on the other hand. Thirdly, the cytological changes which occur in the indifferent sympathetic cell during its differentiation into a medullary cell.

Acknowledgments. For the suggestion of this problem and for helpful criticism, I sincerely thank Dr. B. H. Willier.

MATERIAL AND METHODS

Chicks of from three days' incubation up to three days after hatching were used. The eggs were incubated under optimum conditions. For the earlier stages (three to six days) as well as those of the intermediate stages in which it was desired to study the sympathetic and nervous relationships of the glands, the entire embryo or at least the entire trunk was sectioned. For the cytological studies, only the suprarenals with enough of the surrounding tissue to prevent injury to the glands were dissected out and prepared. In all cases serial sections were made. A total of 160 embryos were prepared.

For general purposes Zenker's, Bouin's, or Tellyesnick's fluids were used. For sensitive tests of the chrome reaction, Regaud's formol bichromate, with neutral formalin, or Orth's fluid modified (potassium bichromate, 2.5 per cent, 20 parts; neutral formalin, 1 part), were most successful. After this fixation the sections were stained with Ehrlich's hematoxylin, without a counterstain. For the Golgi apparatus, Mann-Kopsch's long osmic-acid method, Cajal's uranum nitrate, or his neutral-formalin methods were employed. These methods likewise served for the demonstration of nerve fibers. The mitochondrial techniques employed were: fixation in Regaud's fluid with neutral formalin, or in Helly's formol-Zenker (neutral formalin) followed by osmication in 2 per cent osmic acid for two to three days, gave the best results. The sections were stained with Bensley's aniline-acid-fuchsin, methyl green. A staining method which was frequently used after Zenker's fluid or Bouin's fluid was Masson's hematoxylin-saffranine-erythrosine. This gave splendid differentiation of the sympathetic tissue as well as of nerve fibers in the gland.

ANATOMICAL RELATIONSHIP OF THE SYMPATHETIC NERVOUS SYSTEM TO THE SUPRARENAL BODIES DURING VARIOUS STAGES OF THEIR DEVELOPMENT

1. Initial relationship of the sympathetic cells and the cortical nodules

Origin of sympathetic cell groups at the suprarenal level. Examination of sections of chick embryos of from ninety to ninety-six hours' incubation shows in the anterior portion of the trunk region, or more specifically, from approximately the tenth to seventeenth somite level, segmentally arranged cell aggregations, more closely massed and more heavily staining than the surrounding mesenchyme, lying on each side, dorsolateral to the aorta. The aggregates on each side are connected by loosely interspersed cells, and thus form a sort of chain. They constitute the so-called 'primary chain' of sympathetic ganglia (His, '97). Trains of cells of similar

appearance, lying just posterior to the segmental aortic branches, extend from the spinal ganglia down to these ganglionic masses, and likewise from the masses ventrally, around each side of the aorta into the dorsal mesentery. At the level of the suprarenals, with which we are more particularly concerned, from the seventeenth to the twenty-third somite, and for several somites beyond, the primary sympathetic ganglia are not so prominent. Here the sympathetic cell trains extend from the spinal ganglia, medioventrally to the level of the aorta, and thence ventrally around the sides of the aorta without forming such large aggregations dorsolateral to the aorta.

Due to the alignment of the cells of the trains, to their continuity, and to their morphological similarity to the cells which first appear in the spinal ganglia, it is assumed that they are cells which have migrated by way of the developing spinal ganglia.

From the ganglionic masses of the primary chain while the cells are still apparently in an indifferent stage, cells extend outward ventrolaterally into the loose mesenchyme between the aorta and the wolffian body. Likewise, cells appear to become detached from the cell trains proceeding ventrally from the sympathetic ganglia, as well as from the cell trains of the primary sympathetic tract between the spinal ganglia and the sympathetic ganglia. Apparently these detached cells or cell grouplets are migrating in a posterior direction, since by the end of the fourth day they are found not only in the sections showing the primary sympathetic nerve tracts and ganglia, but also in successive sections between these ganglia and their tracts. Detached sympathetic cells are found in the mesenchyme between the aorta and the wolffian bodies during the fourth day, where they come in contact with the proliferation of coelomic epithelium which is giving rise to the cortical cords of the suprarenal glands. Wherever these indifferent sympathetic cells come into contact with obstructions, such as blood vessels or organs, they collect. Mechanical influences of this nature appear to play a most important

part in the establishment of the permanent ganglia of the surface of the suprarenal bodies, as will be shown later.

Factors concerned in establishment of the sympathetic-chromaffine rudiment. In sections of appropriate levels, namely, from the posterior portion of the seventeenth- to the twenty-second-somite level, the cortical ingrowths are seen proliferating from the thickened coelomic epithelium, which from the twentieth to twenty-second somites becomes continuous with the germinal epithelium. This proliferation produces a series of nodules extending dorsally into the mesenchyme along the medial surface of the anterior portion of the wolffian body. Here they form a ridge about a millimeter in length, which by late on the fourth day has become detached from the coelomic epithelium.

During the entire fifth day, these cortical ridges, one on each side of the aorta, consist of nodules separated by mesenchyme. Crowded by the developing mesonephroi lateral to them, the further growth of the nodules takes place chiefly in a dorsomedial direction. The result is the formation of cortical cords from the nodules. The extension of these cords gives to this portion of the gland the form which it retains to a considerable degree in the definitive state.

At the time the cortical cells are being proliferated from the coelomic epithelium, the first of the indifferent sympathetic cells (sympathogonia, of Poll), which have pushed into the loose mesenchyme from the primary sympathetic cell trains, come into contact with them. As the cortical nodules become larger, and are detached from the coelomic epithelium, they form a ridge lying medial to the mesonephros on each side of the body. The sympathogonia collect over this ridge. At the anterior portion of the ridge they come in contact with its dorsal and medial surfaces and collect in front of its anterior extremity. Over the remainder of the ridge, many of the sympathetic cells remain in contact with it, while others, which remain in the mesenchyme, form trains continuing ventrally. These indifferent sympathetic cells form the rudiment of the sympathetic-chromaffine complex which develops in association with the cortical cords.

The first and most extensive portion of the sympathetic-chromaffine rudiment is at least partially established by the end of the fifth day. This is the 'anterior cap' of indifferent sympathetic tissue.

Due to the fact that sympathetic cells from the primary sympathetic ganglia and from their tracts become detached and are subsequently found in sections posterior to them, these indifferent cells probably reach their final locations at segment levels posterior to the ones containing the tracts from which they originally came. This makes it impossible to state from which particular primary sympathetic ganglion or tract the sympathetic cells of this anterior cap have come. Thus, although the anterior extremity of the cortical ridge at this time lies in the posterior part of the seventeenth somite, it is evident that the primary sympathetic ganglia of more anterior somite levels have contributed to the sympathetic elements around the cortical nodules.

Another feature which brings the anterior ganglia nearer to the region involved is the extreme curvature of the chick on the fourth day. This causes the sympathetic trunks to converge somewhat like the radii of a circle toward their axis. Spinal ganglia which in a day or two will lie considerably farther anterior, due to the elongation and straightening of the embryo, are thus connected with primary sympathetic ganglia lying quite near the suprarenal rudiment.

By 120 hours the cortical bodies occupy more of the free area between the aorta and the wolffian body. Increasing numbers of the sympathetic cells collect on their medial border. At the anterior end of the cortical ridge it is found that the cell trains extending ventromedially in the sympathetic tracts along the posterior surfaces of the dorsal branches of the aorta, come into contact with the more dense mesenchyme around the aorta, turn laterally, and come into contact with the anterior end of the cortical ridge. Here the trains bifurcate. Most of the sympathogonia turn ventrally along the medial surface of the cortical rudiment and become applied to its irregular border. The remainder turn laterally, around

the anterodorsal surface of the cortical ridge, and here form a sort of cap of indifferent sympathetic cells.

The anterior cap of indifferent sympathetic cells, which is laid down from the fourth to the sixth day, constitutes the rudiment of a ganglion as well as of a considerable portion of the prospective medullary tissue which penetrates the gland at a later time. The cap eventually (by the end of the sixth day) covers approximately the entire anterior third of the corticular rudiment, due to the accumulation of additional cells and to the posterior movement of its indifferent cells.

At about one-third the distance of the length of the gland rudiments, reckoning in a craniocaudal direction, lies the next primary sympathetic tract from which cellular elements extend directly to the gangliomedullary primordium. This is the primary sympathetic tract of the twentieth-somite level. As from the preceding one, cellular elements extend from this tract ventrally around the sides of the aorta. At this level the omphalomesenteric artery leaves the aorta and passes obliquely to the right. The aorta wall near the base of the omphalomesenteric artery is stretched to the right, close to the suprarenal body of this side. The subcardinal vein lies ventral and slightly medial to the suprarenal body. The suprarenal body itself slopes ventromedially and appears to become adjusted to the angle formed by the two blood vessels named and the mesonephros lateral to it. The right gland rudiment now lies almost directly in line with the sympathetic cell trains. The indifferent sympathetic cells collect on its medial surface and between it and the aorta and, on its medioventral border, over the subcardinal vein (fig. 1).

The left suprarenal rudiment at the corresponding level lies outside of the direct line of the sympathetic cell trains. No large blood vessels are present on this side. Relatively few sympathetic cells aggregate over this portion of the gland. Later, this is conspicuous as a real difference between the left and the right glands in their definitive form.

These anatomical features are practically identical in each of six five-day chicks, and in each of ten six-day chicks studied

for this purpose. In two of the former and in four of the latter the position of the right gland is slightly more ventral and more nearly in line with the sympathetic cell trains. In each of these a relatively greater number of indifferent cells have accumulated on the dorsomedial surface. In all of the embryos, however, more cells have accumulated on the anterior, medial third of the right gland than on the corresponding part of the left.

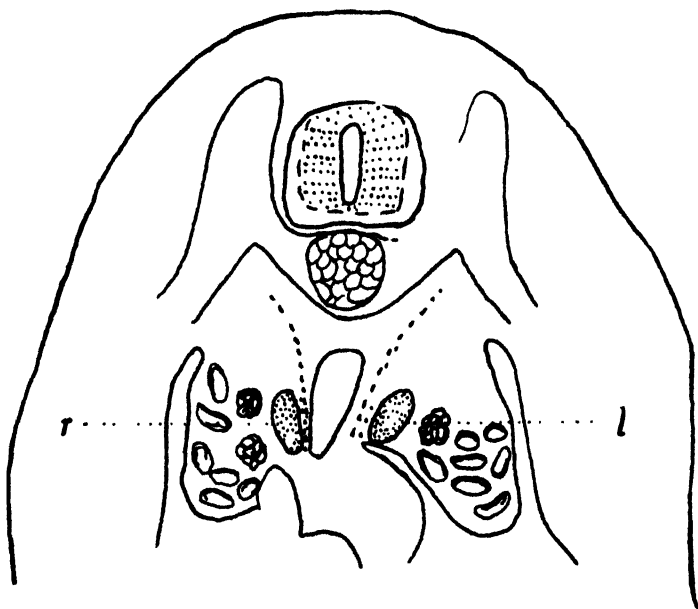


Fig.1 Section through level of twentieth somite of 123-hour chick. Right suprarenal more directly in path of migrating sympathogonia. *r.*, right side; *l.*, left side.

Midway down the length of the glands the sympathetic ganglia of the twentieth-somite level contribute to the pre-medullary components. Here the cell trains on each side divide around an arterial branch which leads from the aorta to the mesonephros, and which lies just dorsal to the cortical ridge. The sympathetic cells course around each side of the artery and become distributed over the medial surface of the cortical body just under it.

About four-fifths of the way down the length of the glands an anastomosis between the right and the left subcardinal veins is established on the fifth day. Over and around this anastomosis the indifferent sympathetic cells collect and form a number of aggregations which are the rudiments of the splanchnic plexus ganglia. These rudiments are in close relationship with the posterior third of the suprarenal bodies. At the time of their establishment, early on the sixth day, there is no apparent difference between the cells applied to the surface of the cortical ridge and a majority of those with which they are continuous in the anlagen of the splanchnic plexus ganglia. A few of the cells of these masses, however, are beginning to show indications of differentiation into ganglion cells.

The development of the secondary sympathetic chain of ganglia begins to take place during the fifth day, and the elements of the primary chain are incorporated into it (Rau and Johnson, '23). The ganglia of this chain are located at or around the bases of the sympathetic trunks and medial to the ventral roots of the spinal nerves. The definitive sympathetic trunks become well established during the sixth day. At the level of the suprarenals they extend ventromedially along the posterior surfaces of the segmental aortic branches. They coincide with the primary tracts about three-fourths of the distance to the original primary ganglia, where they diverge laterally, and lead directly to the sympathetic-chromaffine masses on the dorsal surfaces of the cortical glands.

Cells appear along these tracts which in all particulars correspond to the indifferent cells of primary tracts, and to those around the cortical bodies of the suprarenals. These cells appear to be added to the sympathetic-chromaffine rudiments, since there is obviously an increase in the elements of these masses up to the seventh day. By later differentiation, these cells appear to be capable of forming sympathetic nerve cells as well as chromaffine cells.

On the seventh day important developments occur in the sympathetic-chromaffine rudiments around the cortical

glands. These cell masses have accumulated more heavily at three distinct points, at the anterior end, in the center, and at the beginning of the posterior third of the cortical ridges. They correspond not so much to the migration paths of sympathogonia, but more precisely to the position of the large vessels in close relationship to the cortical bodies. Spread between these masses, but not so heavily as at the points mentioned, sympathetic cells also cover the remainder of the medial surfaces. Between the aorta and the suprarenals, somewhat nearer the aorta, are found sympathetic cells which by methylene-blue, Mann-Kopsch osmication, or Masson's triple stain, are seen to have developed nerve fibers connecting them with the sympathetic trunks leading dorsally to the definitive sympathetic ganglia and ventrally around the aorta to the visceral ganglia. At the posterior level of the suprarenals they lead to the large plexus ventral to the aorta.

Developing between the ganglionic masses on the surface of the cortical gland, and connecting them in the form of longitudinal, anteroposterior chains, are the definitive sympathetic trunks of this level. A trunk connects the ganglionic masses on the mediodorsal surface of each cortical body, another connects those on the medioventral edge. The two chains on each cortical body are connected by dorsoventral connections which lead between the ganglionic masses of the mediodorsal and medioventral ganglionic masses (fig. 2).

Between the sympathetic nerve trunks and the suprarenals are the sympathetic cell groups consisting largely of prospective chromaffine cells. Among the latter, however, are cells which have developed extensive neurofibrils, which from this time on make up a prominent feature of the sympathetic masses. Furthermore, these masses are bounded by, and in close relationship with, other groups whose elements have differentiated into ganglion cells and form the rudiments of sympathetic ganglia. Three distinct cell types are found during the seventh day on the surface of the cortical bodies. They are the ganglionic cells of the rudimentary ganglia, extraganglionic nerve cells, and indifferent sympathetic cells.

RELATIONSHIP JUST BEFORE PENETRATION OF PROSPECTIVE
MEDULLARY CELLS

Innervation of the cortical glands. As early as the fourth day (ninety-six hours), occasional indifferent sympathetic cells may be seen lying among the cortical nodules. Fusari ('93) also noted this. By 120 hours these cells have devel-

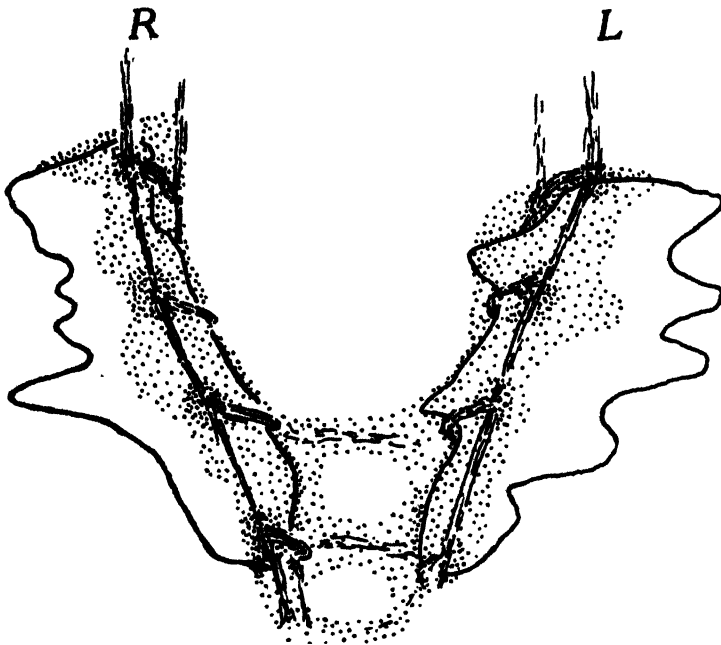


Fig. 2 Reconstruction of chick suprarenals on eighth day, showing distribution of sympathetic components over cortical bodies, and the sympathetic chains on the surface.

oped processes which extend out between the nodules and establish a contact with the processes of other sympathetic cells developing outside of the cortical body, or they may be observed leading directly to the cell masses which are differentiating into sympathetic ganglia on the dorsomedial or ventromedial surfaces of the nodules.

During the fifth day the indifferent sympathetic cells are not invading the cortical mass, since they cannot be observed

in various stages of an actual penetration, as is the case at a later time. On the other hand, there is every indication that the cortical masses have grown up around isolated indifferent sympathetic cells, since such isolated cells are seen in this region at the time that the cortical masses are being proliferated from the coelomic epithelium. At 110 to 120 hours such nerve cells with developing processes can be seen lying in the indentations between cortical nodules, not oriented so as to make it appear that they can be penetrating between the cords (fig. 3). In successive stages, with an extension

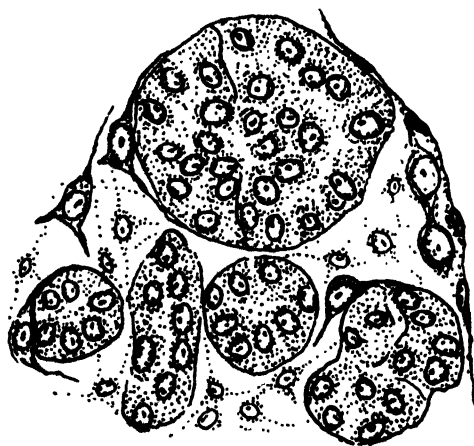


Fig. 3 Section at 120 hours' incubation, showing cortical nodules with sympathetic nerve cells differentiating between them.

of the cortical nodules, the nerve cells appear more numerous in the sections and deeper in the cortical bodies. That the cortical nodules, however, are exhibiting a rapid extension is evident by their increasing mass and by the great mitotic activity of their cells.

Late on the sixth day the nerve cells within the cortical glands have extensive processes. Their processes are closely applied to the cortical cords and others lead outward to the ganglionic masses. Six to eight of these relatively large cells may be observed in almost every section throughout the gland. By the seventh day the multipolar nerve cells within

the cortical glands are better developed. By use of osmic-acid agents, their cell bodies are conspicuous, and a veritable reticulum is formed among the cortical cords by their processes. A distinct nervous association is formed between them and the ganglia lying medial to the gland (fig. 4).

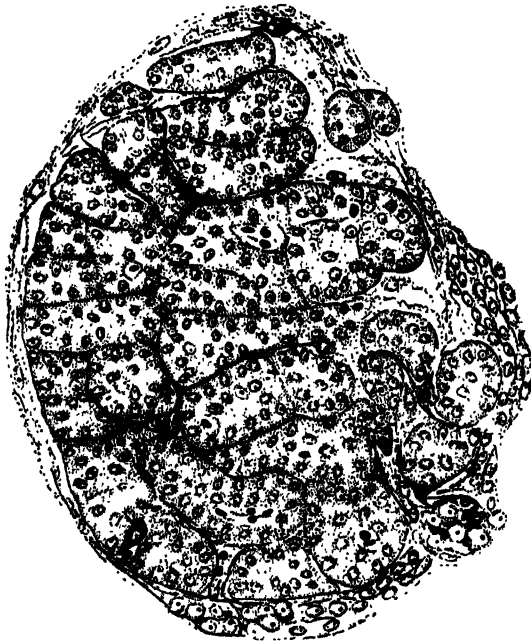


Fig. 4 Section through right suprarenal body early on seventh day, showing multipolar sympathetic cells lying between cortical cords, and the sympathetic-chromaffine complex on the surface of the cortical body.

Innervation of the sympathetic masses. Outside of the cortical body, an extensive nervous network develops during the sixth and seventh days. Making up this network are: 1) Nerve processes which connect the ganglionic masses on the medial surface of the gland, and which form the definitive trunks between the ganglia, and between the two longitudinal chains of ganglia. 2) Nerve processes which lead from the developing ganglia into the groups of indifferent cells. 3) Fibers of sympathoblasts, which differentiate among the

indifferent cells outside of the ganglionic rudiments. The processes of these cells course between groups of indifferent cells and along indifferent cell trains, apparently bringing the indifferent cell groups into association with each other. With the indifferent cells the sympathoblasts make up a sympathetic-chromaffine complex.

From the time that the indifferent cells first collect in the vicinity of the gland to the time at which the chromaffine cells begin their differentiation, there is being established, through the sympathetic cells and their processes, a corticomedullary relationship. This extends from the multipolar cells within the cortical body to the ganglia on the surface of the gland, and from these ganglia to the indifferent cell masses, through the sympathetic network.

The complex is described according to its appearance after the Mann-Kopsch osmic-acid technique, but it is readily demonstrated by the Cajal method or by Masson's triple stain. After the osmic-acid method, the nerve fibers are blackened apparently because a thin layer of myelinated material is being deposited around them. The fibers are not blackened with maximum intensity until the ninth day, but may be followed as early as the seventh day.

RELATIONSHIP DURING PENETRATION OF MEDULLARY CELLS

By the end of the seventh day, nerve fibers passing between the ganglionic masses on the cortical glands are well developed so that these ganglia, and with them the rudiments of the suprarenal bodies, are in connection with the sympathetic trunks. By the middle of the eighth day, which, as we shall see, is the time at which the medullary cells begin to penetrate the cortical glands, the sympathetic system in this region has undergone an extensive development and is nearing its final form. The system at the level of the suprarenals consists of the following parts: first, the secondary or permanent chain of sympathetic ganglia; secondly, a chain of smaller ganglia on the dorsomedial border of the suprarenal, and nerve trunks connecting them; thirdly, a chain of ganglia,

ventrolateral to the aorta and ventromedial to the suprarenals, with connecting trunks, and nerve trunks connecting the dorsal and ventral chains. The ganglia of the ventral chain, toward the posterior end of the suprarenal, are among those of the splanchnic plexus.

The indifferent cell masses, by this time containing many nerve cells and nerve fibers, form a continuous sympathetic-chromaffine mass between the ganglionic groups, around the surface of the cortical bodies.

RELATIONSHIP AT TIME OF HATCHING

The suprarenal glands of the chick at the time of hatching are triangular in outline, about 2 mm. in length, lying at the anteromedial angle of the anterior lobe of the kidneys. The breadth of the gland at the widest point is approximately four-fifths of the length. The thickness of the gland in its center is about one-half the breadth. The broadest part of the gland is directed anteriorly, and its apex posteriorly. The lateral border is irregular and closely applied to the anterior lobe of the kidney. At level 3 (fig. 5), in seven of ten chicks, between ages of eighteen and twenty-two days, the left glands are somewhat flattened dorsoventrally, and this portion of the gland extends farther medially so that a portion of it lies ventral to the aorta. In this respect the gland from which the reconstruction diagrams were made is not quite typical.

Location of the sympathetic ganglia. The sympathetic ganglia regularly found in connection with each of the suprarenals are as follow: the first is an anteromedial ganglion forming a sort of cap over this portion of the gland. At the anterior end the ganglion entirely covers the dorsomedial surface of the gland. In level 1 (fig. 5), the dorsal and the ventral portion of the ganglion are still continuous. Slightly farther posterior, the two parts appear as distinct ganglia. This ganglionic cap over the right gland is larger and more extensive than it is over the left. On the right side the dorsal and the ventral portions of the ganglia extend fully a third of the length of the gland without interruption. On the left

side the ganglion extends no farther posteriorly than the anterior fifth of the gland (level 2, fig. 5). This is probably due to the fact that during the period of cell migration, this gland lies more directly in the path of the sympathogonia, as noted previously.

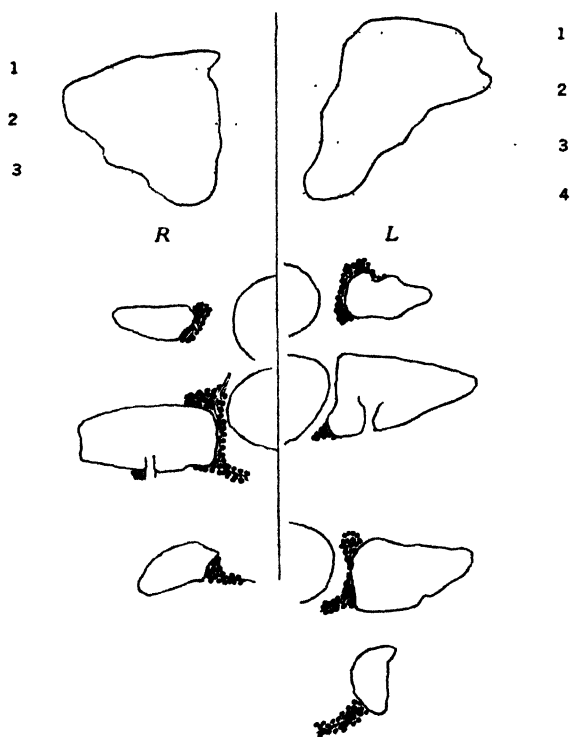


Fig. 5 Reconstruction drawing of suprarrenal bodies on eighteenth day of incubation, with transverse sections through various levels showing position of superficial ganglia. *R.*, right; *L.*, left.

A second, ventromedial ganglion begins just posterior to the first, and extends about one-half the remaining length of the gland. The second dorsomedial ganglion begins several sections posterior to the anterior end of the ventral one, and extends several sections posterior to the end of the ventral one.

During the formation of this ganglionic mass, its dorsal and ventral portions were continuous. After the penetration of the cortical gland by the medullary cells, only the ganglionic portions of the mass remained on the surface of the gland, while the intermediate cells penetrated the gland. A sympathetic trunk remained on the surface which connects the dorsal and ventral ganglia of this pair.

Although ganglia at this level of the right and the left glands correspond approximately in position, those of the left gland are not nearly as large as those of the right. This is again correlated with the previous observation that during the migration period, few sympathetic cells collected over the left gland at this level, for the reasons previously given.

A third pair of ganglia, consisting of a dorsomedial and a ventromedial ganglion, is located on the surface of the posterior third of each of the glands (level 3-4, fig. 5). The ventral ganglia of these pairs is continuous with those of the splanchnic plexus, the ganglia of which lie in the midventral line.

There are only minor differences in the position and the extent of the ganglionic masses described in the ten cases studied. In three of these, a small ganglionic mass is found on the ventral side of the gland, just lateral to the suprarenal vein, on the right side.

From the ganglia and from the sympathetic trunks which connect them, many nerve fibers enter the gland. In fact, the inner portion of the connective-tissue capsule, surrounding the glands, is permeated by a rich supply of nerve fibers, and these are seen to penetrate the gland even in sections which show no ganglionic masses.

These observations are based on the study of serial sections of the glands of twelve chicks, between the ages of eighteen days' incubation and three days after hatching. In all of these, the positions of the ganglia correspond.

CYTOLOGICAL STUDY OF DIFFERENTIATING SYMPATHETIC CELLS

1. The different sympathetic cells

The sympathetic cells which first come into intimate relationship with the cortical nodules are called 'indifferent sympathetic cells,' since it is known from their future development that they must have the potencies of developing into sympathetic nerve cells, or into chromaffine cells. From these cells are formed, A) sympathetic ganglia which remain on the surface of the gland; B) sympathetic nerve cells whose fibers innervate the suprarenal gland and its medullary tissue, and, C) the chromaffine tissue of which the greater part enters the gland as medullary tissue, but of which some remains outside of the gland in association with the sympathetic ganglia.

An analysis of the sympathetic cells of the aggregations around the cortical nodules from the ninetieth to the one hundredth hour of incubation, shows a uniformity of structure. The majority of cells are still apparently indifferent as to their future potencies, so far as structural appearances are concerned. By 120 hours, a considerable number have differentiated to such an extent that they can be recognized as extraganglionic nerve cells or as ganglion cells.

The indifferent cells of the primary tracts, and those lying in the loose mesenchyme, which will later come into contact with the developing cortical nodules, differ visibly from the surrounding mesenchymal cells chiefly in two respects; their cytoplasm in general is more extensive, and in its staining reactions more basophilic than that of the mesenchyme cell. This latter difference is sufficient to contrast them sharply against the more lightly staining mesenchyme. The cytoplasm of the indifferent cells may be drawn out in bipolar form, in which case the extended processes are heavier and thicker than the very fine mesenchymal processes of the mesenchyme cells.

Within the cell trains, the cytoplasm of the indifferent cells is indefinite in extent and has an indistinct margin wherever two cells come in contact. They have a tendency

to fuse more or less completely, as though the cytoplasm had a low surface tension. On the other hand, cells are encountered which extend from the cell trains into the loose surrounding mesenchyme, and others are entirely detached from the cell trains.

Measurements of the indifferent sympathetic cells show that the nuclei have an average diameter of $4.2 \times 4.8 \mu$. The variability from this is slight. The cytoplasm of the cells in the cell trains forms a syncytium in which one cell is continuous with adjacent cells, and consequently cannot be accurately measured. Measurement of isolated sympathetic cells shows that they may be from 8 to 10μ from one extremity to the opposite one. The average width is about 4.3μ . These dimensions remain the same during the fifth and sixth days, until the seventh day, before penetration begins, or up to the time of differentiation of the chromaffine cells, when there is a measurable increase in the cells, the dimensions at that time being $4.3 \times 6.0 \mu$ for the nuclei, and 5 to $5.6 \times 10 \mu$ for the cytoplasm.

The indifferent sympathetic cells of the fourth day show a high rate of mitotic activity, approximately one cell in seventeen showing a division phase. This is the highest rate of mitotic activity exhibited by the indifferent cells during the course of development of the gangliomedullary rudiments. During the fourth day, only occasional sympathetic cells are encountered outside of the cell trains and primary sympathetic ganglia. During the fifth day, more and more of these cells accumulate around the aortic branches, and around the cortical cords, but they also show an increase within the cell trains and in the primary sympathetic ganglia. Probably both the addition of cells from without and their high mitotic rate account for the increased numbers. Cell counts on the fifth day, based on five embryos, show that they have a mitotic rate ranging from 1:50 cells, to 1:130 cells. During the sixth day, not nearly so many mitotic figures are seen. Cell counts again based on five embryos showed the mitotic rate to range from 1:242 cells to 1:643 cells. The variability in the rate

of division is too great to warrant any conclusions as to relative mitotic rate.

2. *The sympathetic nerve cells*

The first visible changes occurring in the indifferent cells of the sympathetic-chromaffin rudiments occur simultaneously in those cells differentiating into sympathetic ganglion cells and in those developing into multipolar sympathetic cells not confined to ganglia, but scattered throughout the sympathetic-chromaffine rudiments and between the cortical cords of the suprarenal. These two cell types may be recognized as early as the sixth day (123 hours), and become quite distinct by the seventh day. The former type of cell in the immediate vicinity of the suprarenal is found to occur chiefly in the loci of the sympathetic-chromaffine rudiments previously designated as centers of aggregation of indifferent cells. During this early period of nervous differentiation (sixth to eighth day), the larger portion of these rudiments is still made up of indifferent cells, but among them sympathetic multipolar cells are differentiating to form a rich nervous network throughout these indifferent cell masses. Within the cortical portion of the gland the indifferent cells which were enveloped by the extension of the cortical cords at this time show the same differentiation.

A. Ganglion cells. During differentiation the nuclei of the indifferent cells show a tendency to become more spherical than the elliptical nuclei which they had as indifferent cells. They appear to be undergoing an enlargement, which, however, does not become conspicuously apparent till the sixth day. The nuclei become clearer and the distribution of the chromatin becomes more uniform. Usually a single large nucleolus becomes more intensely staining and occupies an eccentric position in the nucleus. While the nuclei are undergoing these changes the cytoplasm becomes more basophilic. Cytoplasmic granules become visible around the nucleus. These are especially chromatic to methylene blue. Small grouplets of these cells contrast markedly from the indifferent

sympathetic cells, of the fifth day. By the Golgi methods the rod-like cytoplasmic inclusions around the nucleus become prominent. In addition, a granular, blackened mass usually appears at one end of the nucleus. As the indifferent cell takes the form of a ganglion cell, this mass appears reticular. With the further growth of the cell the reticular body disappears from the polar position and by the fifteenth day lies in the cytoplasm around the nucleus in the form of straight or bent rods. This is the form in which the Golgi apparatus remains in the ganglion cells at hatching (figs. 6, 7, 8).

The mitochondria become most conspicuous in the ganglion cells during the process of differentiation, after these cells have become considerably enlarged (twelfth to fifteenth day). In the indifferent sympathetic cells the mitochondria are small granular bodies lying in the cytoplasm around the nucleus and out in the cytoplasmic extensions. In the ganglion cells they become abundant, though there is a variability in the number in adjacent cells. Here by the Bensley staining method, they are stained bright red. In form they are mostly rod-like, but the rods vary considerably in length. Their orientation in general is parallel to the long axis of the cell, but that this is not necessarily so, is shown by many of the smaller bodies which lie transverse to the longer ones.

From the sixth to the seventh day, by virtue of this differentiation, the ganglia on the dorsomedial and the ventromedial border of the gland become delimited from the mass of indifferent cells around them. By this time the size of the nuclei of the ganglion cells has increased to $8 \times 10 \mu$ in diameter. The changes which began at from twenty-four to twenty-six hours previously have become accentuated. Fully developed ganglion cells which are found by the fifteenth day may have a nuclear diameter of $12 \times 14 \mu$.

B. Multipolar sympathetic cells. The cells between the cortical cords, characterized by their large cell body and multiple processes, by the sixth day appear to become more advanced in their differentiation than the developing ganglion cells outside of the gland. By the Mann-Kopsch technique

the nuclei of these cells by the fifth day show an intensely blackened nuclear membrane. Fine granules appear just inside of this, while in the cytoplasm around the nucleus lie coarse blackened bodies. At one end of the elliptical nucleus is a special mass of blackened granules and filaments, identified as the Golgi material (fig. 9). By the sixth day, the nuclei of these multipolar cells stain brown by the osmic technique. Small vesicles containing black granules have been observed on the nuclear membrane projecting outward into the cytoplasm. The cytoplasmic inclusions have greatly increased by this time (fig. 10). The cell featured in this figure was $8.2 \times 10 \mu$ in diameter.

The Golgi apparatus as in the ganglion cells appears filar and lies farther out in the cytoplasm than it did in the indifferent cells. There is a gradual loss of the Golgi material from the poles of the nucleus and instead it appears in rod-like form in the cytoplasm around the nucleus. In the fully formed ganglion cells the Golgi material appears in the form of twisted rods lying in the cytoplasm, similar to that of the ganglion cell.

3. The chromaffine-medullary cells

Penetration of the gland by the sympathetic-chromaffine complex. The development of the suprarenal from the sixth to the eighth day has been shown to be characterized by an innervation of the cortical cords and of the sympathetic cell masses. The development beginning with the eighth day is characterized by the penetration of the cortical gland by the sympathetic-medullary tissue and the differentiation of the chromaffine cells.

The first indication of penetration appears in embryos of about seven days ten hours (thirty-six embryos). It is first evidenced by the projection between the cortical cords, by strands or trains of cells originating from the sympathetic-medullary complex on the surface of the gland and lying in immediate contact with the gland, extending well in, toward the center. These trains are composed of from five to ten cell

elements. That they are destined to be medullary cells is evidenced by the appearance in some of them of the first positive chromaffine reaction.

When this penetration first becomes apparent, it appears to be due to an amoeboid movement of the cells, in which a single long projection is extended by the cell. The cells are oriented into the intercordal passages. This appears to be similar to the manner by which these same cells arrived in the vicinity of the suprarenal on the fourth day. By the ninth day, this apparent movement of the cells, which can here be observed only by the study of successive stages of penetration, appears to have resolved itself into a general mass movement of the premedullary cells (fig. 11). By 196 hours, large masses of these cells are wedged into the intercordal spaces at the periphery of the gland. At this time all of the cells composing such masses are not oriented in the direction of the flow, as is obvious by the various positions of the elongated cells. Many of these lie transverse to the direction of penetration and appear to be carried along with the general movement of the mass.

Among the penetrating medullary cells the numerous sympathetic nerve cells whose processes ramify among the medullary cells and which help to make up the sympathetic-chromaffine complex also appear to be carried into the gland by the general movement of the cell mass. The nerve cells entering the gland at this time constitute the second group of nerve cells which enter the suprarenal gland, the first having entered as indifferent cells on the fifth and sixth days, while the cortical nodules were being proliferated.

Although the main source of the medullary cells is from the indifferent cell aggregations of the anterior dorsomedial, the central dorso- and ventromedial, and the posterior medial aggregations, the medullary cells do not enter the gland from these loci only, but from the indifferent cells on the surface of the gland between these cell masses as well. Cell trains are seen to extend into the gland from anywhere on the dorsal and medial faces, and from the surface of the gland, where

the indifferent cells were wedged between the cortical cords and the venae cavae. Many of the larger indentations on the surface of the cortical gland contained indifferent cell groups, all of which become centers of penetration.

Penetration appears to begin simultaneously from all parts of the gland surface. It is no more advanced on the dorsal periphery than it is on the medioventral one. Likewise, the strands of medullary cells which extend inward from the ventral side are equally far advanced. The duration of the penetration process is relatively difficult to ascertain, since the medullary cords extend to the surface of the gland even in the definitive condition, and medullary-chromaffine tissue remains around its surface. The definitive condition in the suprarenal appears to be reached, however, by the fifteenth day. By this time the medullary tissue extends throughout the gland, its relative abundance on the surface from this time does not appear to be further diminished. At this time the connective-tissue capsule around the gland also develops. This capsule encloses the ganglia on the surface along with the sympathetic-chromaffine tissue. On the other hand, it excludes some sympathetic-chromaffine tissue from the glands. This remains associated with the splanchnic plexus, and is still abundant here three days after hatching. As late as the thirteenth day, there are comparatively large groups of sympathetic-chromaffine cells at the dorsomedial and the ventromedial borders of the gland, which appear to be reduced considerably by the fifteenth day, while the relative amount of medullary tissue between the cortical cords appears to be increased.

Cytology during transformation. In the differentiation of the indifferent sympathetic cells into chromaffine-medullary cells, several changes occur in the former cells which are probably the result of a single continuous process, the climax of which is the capacity of the cell contents to reduce chromates, or, the 'chromaffine reaction.' This has been shown to be an indication of the presence of adrenaline in the cells (Kingsbury, '11; Ogata and Ogata, '17). The changes taking

place in the cells may be listed as follows: 1) Accumulation of cytoplasmic granules; 2) chromaffine reaction; 3) increase in size of the cells. The succession of events will be described from a single microscopic field in the indifferent cell masses on the surface of the cortical cords, in which various stages of cytological differentiation appear at one time, from the eighth to the tenth day. The differences are identical in each of the preparations made from thirty-two embryos of the eighth day, and thirteen of the ninth day.

Early on the eighth day (174 hours), in the cytoplasm immediately around the nucleus of the indifferent cells, and then in the more peripheral parts of the cytoplasm, faint granules begin to accumulate. These are at first very fine, but soon increase in size and in relative numbers. After fixation in potassium bichromate-neutral formalin, they stain but faintly with the basic stain. After fixation in Bouin's fluid followed by iron hematoxylin, they are more basophilic. After potassium bichromate-neutral formalin fixation, the granules show the faintest trace of a brown shade as early as 174 hours (7J, 7K, 7L). This is, however, too faint and too uncertain to designate as a positive chrome reaction. The granules do not appear to be mitochondrial in nature, since by mitochondrial methods both cytoplasmic granules and mitochondria are visible and show different staining reactions. These cytoplasmic changes are general in the indifferent cells of the medullary rudiment on the surface of the cortical bodies in that they occur simultaneously in different portions of the rudiment; however, not simultaneously in every indifferent cell. The cells showing cytoplasmic granules extend from several sections anterior to the cortical cords (about 70μ) into the indifferent elements of the splanchnic plexus of the posterior third of the cortical bodies. Identical changes occur in the indifferent cells whether they are in immediate contact with the cortical cords or at a distance of 150μ or more medial to them, and at a similar distance posterior to their extremities.

The chromaffine reaction. The brown reaction resulting from the union of chromates and adrenaline has been called 'chromaffine reaction' (Henle, '65). The cells showing the reaction were called chromaffine cells (Kohn, '98), 'chromophile cells' (Stilling, '98), or 'pheochrome cells' (Poll, '06). Kingsbury ('11) and Ogata and Ogata ('17) demonstrated that this reaction in the medullary cells is the result of the reduction of chromates by adrenaline, and that the resulting precipitate was chromium dioxide. They demonstrated that the brown reaction in the medullary cell, after treatment with chromates, indicates the presence of adrenaline, and that the intensity of the reaction may be used as an indication of the amount of adrenaline present. The test has since been used by Weyman ('22) and by Miller ('26) to determine the beginning of adrenaline secretion by the medullary cells in the suprarenals of mammalian embryos, and by Okuda ('28) for chick embryos. In the present paper the reaction is likewise taken as an index of adrenaline secretion by the chromaffine-medullary cells and of the completed stage of differentiation of this tissue.

Toward the middle of the eighth day (180 hours), a faint but distinct and definite brown appearance becomes recognizable in many of the elliptical cells of the medullary rudiments while they are still on the surface of the cortical bodies, and in many of the sympathetic cells farther removed from the glands, namely, 150 μ medioventral from the gland, and the same distance anterior and posterior to it. At the level of the posterior third of the gland, such cells showing the same degree of differentiation have been seen in the root of the dorsal mesentery and at the base of the gonads. The reaction makes its appearance simultaneously, in so far as can be determined, in cells of all parts of the medullary rudiment. The picture which one obtains by examining an entire section of the suprarenal rudiment at this time is that of certain cells scattered here and there throughout the chromaffine-medullary rudiment showing a brown cytoplasm. When more closely examined, however, it is found that stages represent-

ing various phases of differentiation are found in the same microscopic field. These stages range from indifferent sympathetic cells, through cells with cytoplasmic granules of several degrees of accumulation, to cells which show the brown reaction distinctly. Among the latter, there are again several degrees of intensity even at this early period of differentiation.

In the appearance of the chrome reaction, as in the appearance of the cytoplasmic granules, the color first becomes visible around the nucleus, in the region of the heavier granules, and then spreads toward the periphery of the cell. This is usually indicated by a more intense reaction in the granular region about the nucleus. The spread of the reaction throughout the cell is rapid, however, as only a few cells can be found in a section, in which all parts of the cytoplasm do not exhibit the same intensity of reaction.

A definite positive chrome reaction in the suprarenal may be obtained after 180 hours' incubation, by the use of Orth's or Regaud's fluids, modified (see Methods). Each of eight cases after the former fluid and each of two after use of the latter gave positive reactions. At 175 hours, the reaction is too faint to be designated as positive, although cytoplasmic granules are present in many of the chromaffine-medullary cells. Glands fixed in Zenker's, Zenker-formol, or Tellyesnick's fluids, although showing cytoplasmic granules in each of a number of attempts, first showed a definite chrome reaction after ten days. Kingsbury, as well as the Ogatas, noted that both acids and mercury bichloride used with potassium bichromate interfered with the chrome reaction.

By the time the chrome reaction becomes unmistakably apparent, the penetration of the cortical gland by the chromaffine-medullary tissue has begun. Some of the trains of cells which extend inward between the cortical cords show the reaction, while in others it is not apparent, although these particular trains may extend into the gland as far as those in which it is visible. The cells lying adjacent to or between the cortical cords generally show the brown reaction no more intensely than do those lying 50 to 100 μ from the cords.

All of the elements of a train of differentiating chromaffine cells invariably show the same intensity of the chrome reaction, or the same degree of differentiation during the early early stages. This is quite apparent in the relatively long trains between the suprarenals and the aorta. Trains of from twelve to fifteen elements all in identical stages of differentiation are thus frequently encountered. This is likewise true of the trains extending into the gland. Again, groups of five to ten cells, whose cytoplasm is continuous, are encountered, all of which show identical shades of the brown reaction. When these cell trains and cell groups are analyzed, it is found that the processes of sympathetic nerve cells course along at the sides of the train or through the cell grouplets, forming intimate contacts with them. A train of cells was observed along which the nerve fiber did not extend the entire distance. The train did not come into contact with any other chromaffine cell groups in the section, and was several layers removed from the nearest cortical cord. In this train the cells in the center had a pronounced brown color, while those at each end of the train, which were not in contact with the fiber, showed a gradient of brown reaction diminishing toward the free ends of the terminal cells (figs. 12, 13, 14).

Frequent instances are observed in which remotely separated groups of cells all show a pronounced brown color of the same intensity, and these groups of cells are all connected by the same chain of sympathetic fibers (fig. 13). This is most apparent on the eighth and ninth days, when relatively few cells show the chrome reaction, or at the time of their early differentiation.

By the middle of the ninth day (202 hours), an increasing number of the cells of the chromaffine-medullary rudiment have acquired the capacity to reduce potassium bichromate, and the brown reaction has become more intense in others, presumably those which were the first to acquire it. During the succeeding three days this differentiation continues to a marked extent. This is during the period when the sympa-

thetic-chromaffine tissue is invading the suprarenal glands. Cells which are apparently differentiating into chromaffine cells may be found in groups on the surface of the suprarenal until approximately the eighteenth day. It is doubtful, however, whether any strictly indifferent cells are found later than the fifteenth day. By this time all of the cells associated with the chromaffine rudiments are either sympathetic nerve cells or they show granular cytoplasm characteristic of differentiating chromaffine cells. In the medullary cords, however, cells or groups of cells are always found which show the chromaffine reaction to a lesser degree than others, and some which are almost devoid of brown color. These are probably cells or cell groups in different phases of their secretion cycle.

At the time when the chromaffine cells begin their differentiation the ganglionic cells in the ganglionic areas surrounded by the chromaffine-medullary primordium show an advanced stage of development. The ganglia, confined to the definite regions about the gland which were previously described, continue to become more sharply delimited from the eighth to the twelfth days. This is the period in which the chromaffine cells, too, are undergoing their most marked differentiation. During this period it has never been observed that a ganglionic cell gave rise to a chromaffine cell. The differentiation of each cell type is entirely from the indifferent cells which in their early stages (fourth to sixth days) may give rise to either ganglionic cells, or remain indifferent till the eighth day, and become chromaffine cells. Among the latter, however, cells are frequently found which have well-developed processes resembling neuro processes. These were observed by Remak ('47) and by Mitsukuri ('82). The differentiation of sympathetic nerve cells has been described. These likewise are differentiated from the indifferent cells before the chromaffine cells differentiate.

Size increase of chromaffine cells during differentiation. Accompanying the cytological differentiation of the chromaffine cells from indifferent sympathetic cells, there is a gradual

increase in size which in the embryo attains its maximum in chicks just before hatching. Measurement of a large number of indifferent cells of the sixth and seventh days of incubation showed them to be $8.5 \times 5 \mu$. The nuclei are $5 \times 4.3 \mu$. There is no change in their size during this time. At the time the cells show the first secretion granules, just before the appearance of the chrome reaction, they show a slight increase in size, both in nuclear dimensions and in cytoplasmic measurements. Average cytoplasmic dimensions are $10 \times 5.6 \mu$. Nuclear measurements are $6.2 \times 5.3 \mu$.

Little variation is found in cell size of the chromaffine cells during the period between the eighth and twelfth days. Likewise, the variation in size between the smallest and the largest chromaffine cells is small. Most of them conform closely to the measurements given. By the twentieth day many of the chromaffine cells have dimensions of $10 \times 10 \mu$, or of $14 \times 8 \mu$, while the size of the nucleus has not kept pace with the increase in cytoplasm. Nuclear size is only $5.0 \times 3 \mu$, or $6.0 \times 3.5 \mu$. Many of the chromaffine cells are binucleate. In these the nuclei are small and spherical, measuring but $3.0 \times 3.0 \mu$. Some of the cells with two nuclei probably divide secondarily, as cells are found with but a single small nucleus of these dimensions and appearances. The binucleate cells result from a nuclear fission, or irregular mitotic division, which is noticeable at early stages of differentiation.

The shape of the medullary cells varies widely. It is apparently modified by the positions which the cells occupy in the medullary cords. Some of the cells become closely applied to the cortical cords and become crescent-shaped. Others remain long and spindle-shaped as they were during early stages of differentiation. Still others are oval. After they become chromaffine cells, their cell boundaries become more definite and distinct than they were in the indifferent stage or in early stages of differentiation.

Golgi apparatus of chromaffine-medullary cells. The suggestion that the Golgi apparatus might afford differences in the premedullary cells by which they could be distinguished

at an early time offered itself. Further, the fact that the Golgi apparatus has been demonstrated to occupy a more or less definite position, in the more active region of secreting cells, suggested that it might be taken as an index of cell polarity during penetration of the cortical gland by the chromaffine-medullary cells. Rau and Ludford ('25) had, however, demonstrated that the Golgi apparatus underwent little change in form during the differentiation of chromaffine cells, but underwent considerable change in the differentiation of nerve cells from the same type of cell from which the chromaffine cells originate. Pilat ('12) showed that the medullary cells occupied no fixed polarity with regard to any fixed structures in the suprarenal glands of porcupines, with the position of the Golgi body taken as an index of the cell's polarity.

The Golgi apparatus in the cells of sympathetic trains of the fifth day, and in sympathetic cells around the cortical nodules, consists of more or less scattered material lying in the cytoplasm close about the nucleus, and a more dense mass of blackened material localized at one nuclear pole. The mass appears to consist of an aggregation of granules. The characteristic network is not evident in these early stages. By the seventh day, the Golgi apparatus of the chromaffine-medullary cells beginning to penetrate the suprarenal appears most frequently in a more definitely organized network, lying close to the nucleus at one pole. The transition to this form from that of the earlier sympathetic cells appears to be a spreading out of the blackened material along definite lines in the cytoplasm, so that it appears in the form of rather closely wound loops. Frequently it covers a small portion of the nucleus. In the cells of the trains extending into the gland, the apparatus is found at the anterior pole of these cells (figs. 15, 16). By the eighth day, when the mass movement of the chromaffine-medullary cells into the cortical gland begins, many cells appear to be carried into the gland with the general movement. Here the position of the Golgi apparatus is still a polar one within the cell, but the pole con-

taining the apparatus is not necessarily foremost with reference to the line of penetration. The pole of the cell containing the apparatus during penetration may be directed away from the gland.

In the suprarenal glands of chicks of twelve to fifteen days, in which the invading chromaffine cells have become stationary, the apparatus retains its position at one pole of the nucleus. Adjacent cells within a cell group, however, are oriented in different directions. There does not appear to be a group polarity, but the polarity of each cell appears independent of the adjacent cells. The cells are not oriented in any definite manner with respect to the cortical cords. In cells in contact with the cortical cords, the apparatus is sometimes directed toward the latter, away from it, or so as to indicate a lateral position of the cell with respect to the cortical cord. The position of the apparatus within the cell is fairly constant, but the orientation of the latter has no relationship to the cortical cords (fig. 17).

The Golgi apparatus does not show quite the same form in all of the chromaffine cells. There are gradations ranging from a granular mass in which no vacuoles or vesicles appear, through a more organized arrangement where the blackened material has the form of a closely wound skein in which smaller and larger vesicles appear. These differences appear both after Mann-Kopsch and after the Cajal methods of preparation, so that it may not be altogether due to the blackening of various elements of the cell. The changes are suggestive of physiological phases of the secretion process evidently taking place in the cells.

Mitochondria of the chromaffine-medullary cells. There is little to be found in the literature regarding the mitochondria of the medullary cells. Mulon ('10) described three types of mitochondria in the medullary cells of rabbits and of guinea-pigs. These types were: first, rather long flexible rods occupying a juxtanuclear position and others of the same type interwoven in the center of the cell; secondly, rodlets shorter than those of the first type, and more scattered in the cyto-

plasm; thirdly, granular mitochondria, scattered in the cytoplasm. He regarded these types as transitional between the first and third types, and the latter as giving rise to the chromaffine granules. DaCosta ('13) does not agree with Mulon regarding the shape and distribution of the mitochondria, from a study based on a number of animals. He finds only the granular type, and apparently finds them less numerous than did Mulon. According to DaCosta, they occupy a peripheral position in the cell.

In this work, the mitochondria of the indifferent sympathetic cells are found to be of the small granular type. These bodies are slightly elongate and lie parallel to the long axis of the cell. They are in rather close apposition to the nucleus in these cells, excepting at either end of the nucleus, where the cytoplasm is more abundant. Here they extend out into the cytoplasm away from the nucleus. They occupy this position during the penetration of the cells into the gland (fig. 18). As differentiation of the cells into chromaffine cells progresses, and as the cytoplasm becomes more abundant, the mitochondria are less numerous immediately around the nucleus, but instead, are found to lie more peripheral in the cytoplasm (fig. 19). Stained with acid fuchsin, they are not likely to be confused with chromaffine granules, as is the case after staining with iron-hematoxylin. At the time of hatching they are best seen in the chromaffine cells with a clearer cytoplasm and fewer granules, or in the cells regarded as having emptied their secretion. The nuclei of the chromaffine cells, by the mitochondrial staining methods, may either differentiate as clear vesicles, in which few chromatin granules are distinguishable, or as solidly stained bodies which do not differentiate. DaCosta described these types for the medullary cells of birds and mammals.

DISCUSSION

Developmental mechanics of relationship between sympathetic cells and cortical cords

These relationships owe their origin to the aggregation of indifferent sympathetic cells on the dorsal and medial surfaces of the cortical nodules. The establishment of the aggregations in this position appears to be largely circumstantial, determined, first, by migration of indifferent sympathetic cells along the surfaces of blood vessels to the site of the cortical nodules, and, secondly, to impediments which the migrating cells encounter.

The migration of the indifferent sympathetic cells ventromedially along the posterior surface of the dorsal segmental arteries takes place at all somite levels. Arriving at the dorsolateral surface of the aorta, the cells encounter their first obstruction and form a series of aggregations, which are the primary sympathetic ganglia. From these aggregations cells continue ventrally. Anterior to the cortical nodules, where no obstructions are encountered lateral to the aorta, they continue ventrally through the dorsal mesentery without forming other aggregations. At the suprarenal level, on the other hand, where the cortical nodules become obstructions in the path of the cells, aggregations are formed on the surface of these bodies.

The location of the aggregations on the surface of the gland is again determined by two major factors which are: A) The position and form of the gland, and, B) the position of blood vessels in this level.

At the anterior extremity the conditions are the same in both cortical bodies. Sympathetic cells pushing out from the cell trains are apparently obstructed by the bodies and collect in front of them. The anterior cap of indifferent cells which is thus formed is identical in each of the bodies. Slightly farther posterior, where the aorta wall is stretched toward the right, near the base of the omphalomesenteric artery, the sympathetic cell trains extending ventrally from the primary sympathetic ganglia, come into closer contact with the right

cortical ridge than with the left. The consequence is that more sympathetic cells aggregate on the right gland at the level of the omphalomesenteric artery. At the same level on the left gland, only a small arterial branch appears as an obstruction, over which relatively few sympathetic cells aggregate. The result is a permanent difference in the size of these ganglia in the definitive organs at this level.

Over the posterior third of their medial borders, the sympathetic cells aggregate over the subcardinal veins and establish the rudiments of permanent ganglia in contact with the ventromedial border of each cortical body.

The effect of the form of the gland on the establishment of permanent ganglia is expressed at the twentieth-somite level of the right body, where sympathetic cells aggregate over the flattened dorsomedial border, and become the rudiment of a relatively large ganglion. In the same level, the left gland is not flattened dorsoventrally, and retains few of the sympathetic cells which come in contact with it. Farther posterior, however, the left gland projects medially under the aorta and has a gradual slope. Here large masses of sympathetic cells are retained on its surface.

In addition to the factors named, it is probable that thigmotaxis, by which the indifferent cells tend to follow surfaces, plays a part in the distribution of these cells over the surfaces of the cortical ridges. In the first place, the sympathetic cells arrive at the site of the suprarenals by following the surfaces of the segmental arteries. Then, again, they follow the surface of the cortical ridges, filling indentations, and remaining in depressions and angles. Many push away from the original aggregations and become rather evenly distributed and more closely applied to the surface of the cortical bodies between the aggregations.

The importance of blood vessels and such mechanical factors as flexures have long been recognized as important in the establishment of ganglionic rudiments. Wm. His, Jr. ('93, '97), emphasized their importance in the location of cervical ganglia, where the numerous flexures form mechani-

cal obstructions in the path of migrating sympathetic cells, and in the abdominal region where the veins of the post-cardinal system obstruct these cells. Fusari ('93) noted that the ganglia of the solar plexus formed around the anastomosis of the cardinal veins. He did not point out the influence of the various vessels in the localization of each of the ganglia around the cortical rudiments, although he described the early relationship of the sympathetic elements to the cortical rudiments. Wiesel ('00) regarded the early relationship between the sympathetic cells and the cortical rudiments in the pig as circumstantial, although he did not point out the particular influence determining the localization of individual ganglia.

Innervation of the suprarenal rudiments

The first sympathetic cells to be found among the cortical nodules may be recognized as early as ninety-six hours. Fusari ('93) observed these, and regarded the penetration of the sympathetic cells into the cortical rudiments as a continuous process beginning at this time. He did not recognize the real active penetration which begins on the eighth day. Goormaghtigh ('21) described a double penetration of the gland. The first of these, beginning at four days sixteen hours, he considered to be responsible for the innervation of the gland. Harris ('28) likewise saw nerve cells in the gland at 144 hours, and assumed that they had penetrated previous to the sixth day, and that they were responsible for the innervation of the gland. The degree of differentiation attained by these cells prior to the penetration of the chromaffine cells was not described by these authors.

This 'penetration,' as described in the present paper, is seen to be a penetration not in the sense of an active movement into the gland by the indifferent cells, but is due more directly to the proliferation and the extension of the cortical cords around certain cells of sympathetic origin, found in contact with the cords. Goormaghtigh was partially correct in regarding them as providing the innervation of the gland;

however, they are not responsible for the entire innervation of the suprarenal, since large numbers of nerve cells are later carried into it by the invasion of the sympathetic-chromaffine complex. The first sympathetic cells to differentiate among the cortical bodies before penetration of the gland by the sympathetic-chromaffine complex are a part of the extensive nervous reticulum which is likewise developing in the sympathetic-chromaffine masses.

The profuse innervation of the cortical tissue as well as of the prospective chromaffine tissue prior to the differentiation of the latter has not been stressed. Various authors have emphasized the abundant nerve endings in the medullary tissue of the suprarenals of various animals in their definitive form, but not in their rudimentary form. Remak, as early as 1847, recognized an embryological relationship between the glands and the sympathetic system. Mitsukuri ('82) likewise noted the abundant nerve supply developed in the chromaffine tissue in the suprarenal gland of rabbit embryos, and traced nerves from the medullary tissue within the gland to the ganglia on the surface of the gland. He found the medullary tissue to be derived from the sympathetic cells. Whitehead ('03) described a "chain of ganglia on the dorsal surface of the adrenal of the pig, and another along its ventral surface," similar to those described in the present paper for the chick suprarenal. He did not regard the nerve trunks as having any significance to the gland itself, and stated that there was no reason to regard them as anything but nerves to adjacent anlagen. Abel ('12), however, noted a rich nervous development between the aorta and the cortical body in the chick during the seventh day, as well as delicate outgrowths extending between the groups of sympathetic cells. Very recently, Pankratz ('31), considering a possible correlation between the beginning of foetal movements and adrenaline secretion in the white rat, observed a rich nervous development in the prechromaffine tissue, and designated the masses as a 'sympathochromaffine complex.'

According to the present paper, there is a profuse innervation of the sympathetic-chromaffine masses around the cortical rudiments, on the seventh day. During this time the definitive sympathetic trunks become well established in this region and many small nerve cells extend their fibers through the indifferent cell masses, until no groups of cells remain isolated. Furthermore, the nervous network which develops among the cortical cords is continuous with the reticulum which develops outside of the cortical bodies. This network attains its maximum development by the end of the seventh day, several hours before the chromaffine cells show any indications of differentiation. This sympathetic mechanism appears, morphologically, to be capable of integrating the entire complex.

Differentiation and penetration of the chromaffine-medullary cells

The following questions now arise: Is it not possible that, under stimulation through the splanchnic nerves, processes are set up in the indifferent cells which are climaxed by the secretion of adrenaline? Moreover, since the process of differentiation begins at approximately the time that penetration of the gland by a portion of the sympathetic-chromaffine complex begins, and since the chromaffine differentiation occurs in the general vicinity of the suprarenal gland, might not the original stimulus responsible for differentiation originate in the cortical portion of the gland?

The fact that distantly isolated strands or groups of cells, which, however, have a direct nervous connection with each other, through the sympathetic fibers, show an equal degree of differentiation, is striking. Although not all of the pre-chromaffine cells differentiate into chromaffine cells at the same time, it appears that differentiation of the cells from indifferent cells is inaugurated over a rather short period of time. After the twelfth day, cells in the sympathetic-chromaffine aggregations, which do not show a distinct brown reaction, are nevertheless granular, and apparently in the

process of secretion. They no longer appear like the indifferent sympathetic cells of the early seventh day.

If adrenaline secretion is initiated by nervous stimulation, then the origin of this function appears to resemble the physiology of its secretion in the adult stage, since it is well known that stimulation of the splanchnic nerves increases the adrenaline output of the gland, and that adrenaline secretion, in general, is under the control of the sympathetic nervous system.

Van Campenhout ('27) described a relationship between sympathetic fibers and the primary insular cells of the pancreas of various mammals, similar to the sympathetic-chromaffine complex described in this paper. It was shown by him that the first insular cells to differentiate were always in contact with sympathetic fibers which developed from sympathoblasts in the vicinity. More recently, however, the same author (Van Campenhout, '30) emphasized a differentiation of chromaffine cells in amphibia as taking place before the sympathetics have developed sufficiently to play a part in this differentiation.

It is apparent that the differentiation of the chromaffine cells is not due to immediate contact of prechromaffine and cortical elements, nor to proximity of individual indifferent cells to the cortical cords. The only connection between these two tissues is through the sympathetic network. If the cortical cells do offer a stimulus, the network must be the means of conducting it, since this is the only connection between the two issues. Even so, it might be expected that the indifferent cells bordering the cortical cords, or those lying between them, would be more favorably situated, and would show the reaction earlier and with greater intensity than the more remote cells. It has, however, been shown that this is not the case. This fact has also been emphasized by Miller ('26) for the mouse.

Miller (p. 272) explains the differentiation of the chromaffine cells "in an apparently homogeneous group," on the assumption of "something inherent in the cell, rather than to

its environmental conditions as the cause of this specialization." This statement is interpreted to mean that certain sympathetic cells have the specific value of chromaffine cells prior to their differentiation. This specific value might be due either to qualitative differences in the hereditary mechanism or to cytoplasmic differences in the cells. The first of these explanations is untenable according to the modern view of the cause of cellular differentiation. The alternative one merely presupposes a determination at an earlier time in the ontogenetic history, but, nevertheless, it must still depend upon environmental factors, acting upon a definite hereditary complement.

The sympathetic-chromaffine complex described in this paper is a mechanism which affords sufficient means for the expression of environmental effects upon indifferent sympathetic cells, namely, stimulation through the sympathetic nervous system. Yet, the possibility that the activity of the cortical cells offers such a stimulus, in view of the nervous relationships, is not eliminated. The findings of Willier ('30) are especially suggestive of such a possibility. In chorio-allantoic grafts he observed an especially close relationship between the medullary cells and the cortical cords. It would be interesting to note whether the nervous development in such grafts approaches that of the normal.

Concerning the 'delayed penetration' of the chromaffine cells between the cortical cords, first described by Soulie ('03), the following questions arise: Why do sympathetic-chromaffine cells which have lain in juxtaposition to the cortical body for several days penetrate between the cortical cords, and, the converse of the question, why do the sympathetic-chromaffine cells lie as a contiguous mass on the surface of the cortical body for several days before penetrating between the cords? To these questions we have no answer. The method by which the cells penetrate the gland may, however, be analyzed in this connection. Whitehead ('03), working on the pig, and Hett ('25), on the human embryo, state that the cells appear to follow a course of least resistance during

their movement into the gland, which Hett describes as amoeboid. If the movement, however, were entirely a random, amoeboid one, and if the individual cells followed the course of least resistance, the cells would not enter the gland at all, in the case of the chick, but would merely move around its sides, since the connective-tissue capsule does not inclose the gland till after the fifteenth day. Furthermore, the chromaffine cells lying at a distance from the cortical cords apparently show no migration or movement at this time. They continue to occupy the same relative position at the sides of the aorta and around the splanchnic plexus. Only the cells immediately around the cortical cords appear to be concerned in the penetration movement.

In this paper the penetration is described as a mass movement by the adjacent sympathetic-chromaffine cells, rather than as a movement of separate, individual cells. The complex as a whole shows successive stages of penetration. Many of the individual cells within the cell group are not oriented with their long axes parallel to the line of penetration. For this reason it is conceivable that the cortical cords may attract the adjacent cell complex. No suggestion can be made, however, as to the nature or mechanics of such an attraction. We do know that mass movements of cells occur, for example, in Triton embryos. The mechanics of these mass movements are likewise obscure at present.

SUMMARY

1. A study was made of the development of the suprarenal gland of the chick with reference to the following points:
 - 1) Topographical relationship of the sympathetic cell masses to the cortical cords and to the sympathetic nervous system.
 - 2) Cytological differentiation of the sympathetic cells.
2. The aggregation of indifferent sympathetic cells, although general over the entire medial surface of the cortical bodies, is more pronounced in the following regions:
 - 1) Over the medial border of the anterior end.
 - 2) In the middle of the gland, around mesonephric branches of the aorta, and on

the right side in the angle between the omphalomesenteric artery and the cortical body. 3) Along the medial surface of the posterior third of the gland, over the subcardinal veins, and over an anastomosis between them.

3. A dorsomedial and a ventromedial chain of ganglia develop on the surface of each gland. The location of the ganglia of each chain corresponds in position to the aggregation enumerated above. The indifferent cells between the aggregations penetrate the gland during differentiation of the medullary tissue.

4. Development in the gland during the sixth and seventh days is characterized by the differentiation of nervous tissue. The ganglia show marked development, the ganglionic cells developing from indifferent cells of the aggregations. Within the sympathetic cell masses many indifferent cells differentiate into nerve cells whose fibers innervate these rudiments. All cell groups of the rudiments are thus brought into association with each other.

5. At the same time, sympathetic cells which were inclosed between the cortical cords during the extension of the latter early on the fifth day, differentiate into nerve cells, whose fibers innervate the cortical body. By the seventh day, the fibers of these cells have also established association with those of the cell masses on the surface of the gland.

6. Differentiation of the 'chromaffine cells' begins early on the eighth day. During the process, cells which were formerly indifferent sympathetic cells show an accumulation of cytoplasmic granules, and an increasing tendency to show the 'chromaffine reaction.' The reaction becomes distinct in some cells at approximately 180 hours.

7. During differentiation, chromaffine cell groups having a direct nervous association with each other show identical stages of differentiation, as indicated by the intensity of the chrome reaction. No direct relationship is noted between intensity of reaction and proximity to the cortical cords.

8. Cytoplasmic inclusions, namely, Golgi apparatus and mitochondria in the chromaffine cells during differentiation, show little change in form from those of the indifferent cells.

9. Penetration of the chromaffine-medullary cells between the cortical cords is inaugurated at the same time that differentiation first appears. Except in early stages, penetration appears as a mass movement of cells rather than as an amoeboid movement of individual cells. The nerve cells of the cell masses are also carried into the gland by the movement.

10. In consideration of the intimate nervous associations between cell groups of the sympathetic-chromaffine complex at the time of differentiation of 'chromaffine cells,' and in view of the observation that directly associated cell groups show the reaction simultaneously and with equal intensity, it appears reasonable that differentiation (adrenaline secretion) may be directly dependent upon the sympathetic system. In view of the nervous associations between the cortical cords and the sympathetic-chromaffine complex, the fact that chromaffine-cell differentiation occurs in the general vicinity of the cortical body, and begins simultaneously with the penetration of the latter by the differentiating medullary cells adjacent to the cortical body, a dependence of this differentiation upon the cortical cells is not inconceivable.

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PLATE

PLATE 1

EXPLANATION OF FIGURES

6 to 10 Differentiating nerve cells. Figures 6, 7, 8, cells from ganglia on surface of cortical body from seventh to twelfth days, showing Golgi material. Figures 9 and 10, multipolar cells lying between cortical cords prior to eighth day.

11 Sympathetic-chromaffine complex during its penetration between cortical cords on ninth day.

12 Sympathetic association between cell groups of sympathetic-chromaffine complex on eighth day. Differentiation of chromaffine cells.

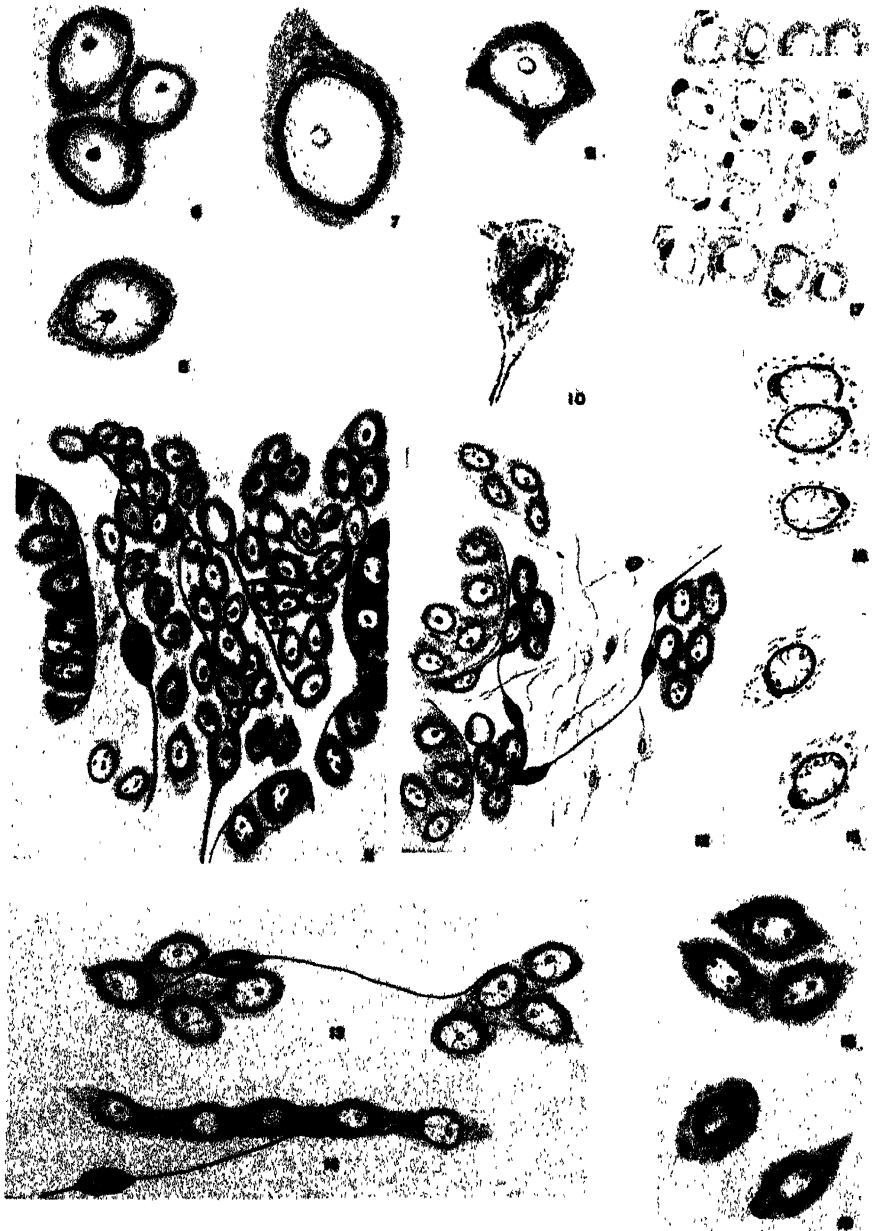
13 and 14 Sympathetic associations with differentiating chromaffine cells on ninth day.

15 Golgi apparatus of indifferent cells, eighth day.

16 Golgi apparatus of differentiating chromaffine cells.

17 Various orientations of chromaffine cells lying between cortical cords on tenth day. Golgi apparatus taken as index of cell polarity.

18 and 19 Mitochondria of indifferent cells and of chromaffine cells, respectively.



THE CHONDROCRANIUM OF A 23-MM. NECTURUS¹

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NINE FIGURES

AUTHORS' ABSTRACT

This study is based on a wax model reconstructed from serial sections stained with basic fuchsin and picro-indigo carmine. It shows: 1) that the columna ethmoidalis and planum basale are chondrified at this stage; 2) that a cartilaginous antorbital process is present; 3) that the stapedial plate is cut out from the wall of the otic capsule; 4) that the so-called crista trabecularis is an independent element homologous with the sphenolateralis of the Anura; 5) that the fourth visceral arch is not present and that the fifth is still in the precartilaginous stage; and, 7) that there is no evidence that a pro-occipital vertebra is formed.

FOREWORD

In connection with other problems in the embryology of *Necturus maculosus*, a wax model of a 23-mm. larva was reconstructed by the junior author. Later study of this model, and the slides from which the reconstruction was made, revealed certain points not hitherto mentioned by investigators in this field. It seems advisable, therefore, to put on record an account of the anatomy of the skull at this stage of development.

The larva from which the reconstruction was made is one of a series collected by Dr. A. R. Cahn at Oconomowoc, Wisconsin, in 1924. After fixation in Bouin's picro-formol-acetic, the greatest length was 23 mm., and comparison with Eycleshymer and Wilson's normal tables indicates an age of fifty-five days. Paraffin sections cut at 20 μ were stained in the senior author's modification of Cajal's fuchsin and picro-indigo-carmin. This stain gave a brilliant differentiation of the various tissues according to the accompanying table.

¹ Contribution from the Zoological Laboratories, University of Illinois, no. 424.

Cartilage	Light pink
Muscle	Bright green
Glands	Light blue
Precartilage	Medium blue
Blood	Medium green
Connective tissue	Purple
Yolk	Olive
Bone	Dark blue
Nerves	Light green

The sharp color distinctions between cartilage and precartilage, muscle, and bone were particularly helpful in the subsequent reconstruction. This was made by Born's method at a magnification of $66\frac{2}{3}$, so that the completed model was 36 cm. in length.

DESCRIPTION OF THE CHONDROCRANIUM

In the following description the term neurocranium is used to indicate the parts of the chondrocranium associated with the brain and sense organs, while the term splanchnocranium is used to include the derivatives of the visceral arches.

The notochord, which is used as the principal point of reference, is practically straight and extends forward in the midline of the neurocranial floor as far as the hypophysis cerebri. A distinct cap of cartilage covers the anterior end of the notochord.

The parachordals (figs. 4, 5, and 9a, *pc*) are flattened and diverge widely at their anterior ends. Posteriorly they widen and approach the notochord. They extend from the level of the hypophysis to that of the foramen of the perilymphatic duct. At the anterior end they are fused with the trabeculae. They are united with the floors of the otic capsules at their outer margins, and posteriorly are connected with the basal plate.

The rod-shaped trabeculae (figs. 1, 2, 3, and 9, *t*) extend from the parachordals to the anterior tip of the head, slightly beyond the forebrain. For two-thirds of their length they extend forward parallel with each other, but thereafter converge slightly to their anterior extremities. They end as free processes in the connective tissue which surrounds them.

Near the anterior ends of the trabeculae is a cartilaginous plate, the planum basale (figs. 1 and 9a, *pb*), which connects the two bars. Its anterior extremity is directly below the anterior point of the forebrain. This plate is trapezoidal with

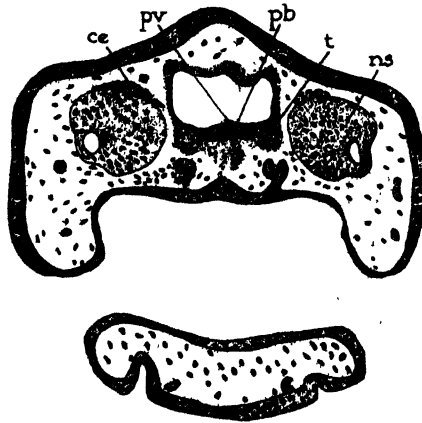


Fig. 1 Cross-section through the anterior portion of the planum basale (*pb*), showing its relation to the two trabeculae (*t*). The slight dorsal swelling of the planum basale is the planum verticale (*pv*). The columna ethmoidalis (*ce*) is dorsal to each nasal sac (*ns*) and is surrounded by a considerable amount of precartilage.

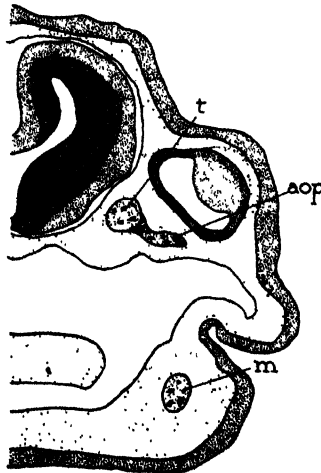


Fig. 2 Cross-section through the anterior part of the antorbital process (*aop*), showing its relation to the trabeculae (*t*). The two rods of Meckel's cartilage (*m*) are ventral to the structures of the neurocranium.

its greater width at the posterior end. As yet, there is some precartilagae connecting it posteriorly with the trabeculae. The anterior coalescence of the two is complete. A median swelling upon the dorsal surface of the planum basale represents the planum verticale (fig. 1, *pv*), which together with the trabecular swellings gives the plate a biconcave appearance. The olfactory lobes of the forebrain rest in the concavities so formed.

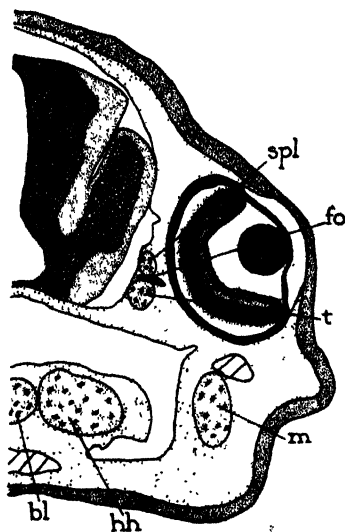


Fig. 3 Cross-section through the region of the optic foramen (*fo*), showing the relation of the sphenolateralis (*spl*) to the trabecula (*t*). The structures of the splanchnocranium which appear in this section are the first basibranchial (*bl*), the hypophyal (*hh*), and Meckel's cartilage (*m*).

Lateral and slightly dorsal to the anterior part of each trabecula is a flattened cartilaginous bar, the columna ethmoidalis (figs. 1 and 9, *ce*). This is dorsal to the nasal sac and is the only part of the nasal capsule chondrified at this stage of development. It extends from a region opposite the middle of the planum basale to the tips of the trabeculae, above the anterior two-thirds of the nasal sac. There is no point of connection between it and the other parts of the chondrocranium.

Slightly in front of the point of convergence of the trabeculae and fused to the outer side of each are two triangular-shaped projections, the antorbital processes (figs. 2 and 9, *aop*). There is some evidence that these processes arise independently of the trabeculae, since at the point of coalescence of the two there is considerable precartilage, especially at the anterior part. Chondrification is apparently taking place from the outer portion of the projection toward the trabecula. The anterior side of the antorbital process is perpendicular to the median plane of the neurocranium. The lateroventral side slopes abruptly from the outer point of the anterior side to the trabecula. The antorbital process extends forward to the posterior end of the nasal sac, below and slightly behind the most anterior point of the eye. It extends under the eye a distance approximately one-tenth of the diameter of the eye.

The sphenolateralis (figs. 3 and 9, *spl*) is a rod-shaped cartilage lying dorsal to each trabecula. These two structures are joined at three points. The most anterior is formed by the direct coalescence of the sphenolateralis with the trabecula. The line of fusion at this point is of greater length than that of the other two, the sphenolateralis gradually merging into the trabecula. The farthest point forward at which it is possible to distinguish it as a separate and distinct process is halfway between the posterior end of the antorbital and the anterior end of the foramen opticus. The second point of junction is made by a very short cross-bar, as is the third. The distance between these two bars is three times that between the first and second points of juncture. Thus, between the three points, two foramina are formed. The anterior and smaller of these, the foramen opticus (figs. 3 and 9b, *fo*), affords passage for the optic nerve. The posterior and larger, the foramen oculomotorious (fig. 9b, *foc*), gives passage to the oculomotor nerve. From the posterior cross-bar, the sphenolateralis continues back, fusing with the dorsal part of the ascending process of the quadrate. It continues a short distance beyond this in the direction of the otic capsule, ending bluntly in the surrounding connective tissue, free from the other cartilages of the chondrocranium.

The basal plate (fig. 9a, *bp*) is one of the two cartilaginous structures of the neurocranium which gives a continuous floor to the cranial cavity. Its sides are fused to the floor of the otic capsule. In the center there is a groove in which the notochord (*nc*) lies. The anterior part of the plate coalesces with the parachordals, the posterior with the base of the occipital arch. The basal plate with the two parachordals, the

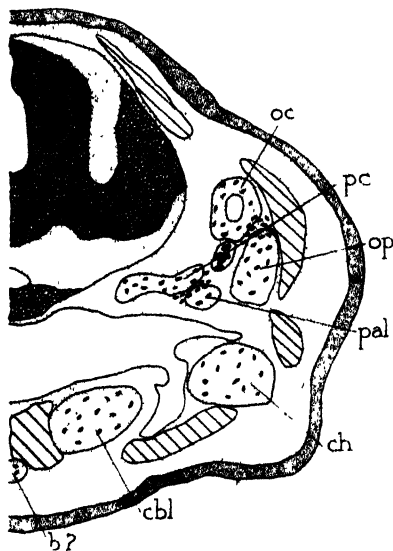


Fig. 4 Cross-section through the anterior tip of the otic capsule (*oc*), showing the relation of the otic process (*op*) and the palatobasale (*pal*) of the quadrate to the capsule. The parachordal (*pc*) is wider than the trabecula which had fused to it anteriorly. The first ceratobranchial (*cb1*), second basibranchial (*b?*), and the ceratohyal (*ch*) can be identified in this section.

two trabeculae and the planum basale completely encloses the largest foramen of the neurocranium, the basicranial fontanelle.

The floor of each otic capsule (figs. 4, 5, and 9, *oc*) is a lateral continuation of one of the parachordals and the basal plate. In this stage of development, the capsule almost encloses the auditory vesicle. Its long axis is parallel to the notochord. In cross-sectional view (fig. 5) it is almost triangular in shape. The inner wall is perpendicular to the

floor, thus separating the brain and the auditory vesicle. The floor continues laterally from this inner side, rising slightly at its midpoint to meet the third side. The third, or dorso-lateral, side completes the triangle, as the hypotenuse of the right angle formed by the inner side and floor of the otic capsule.

There are seven foramina which permit the passage of blood vessels, nerves, and ducts to and from the ear. Four

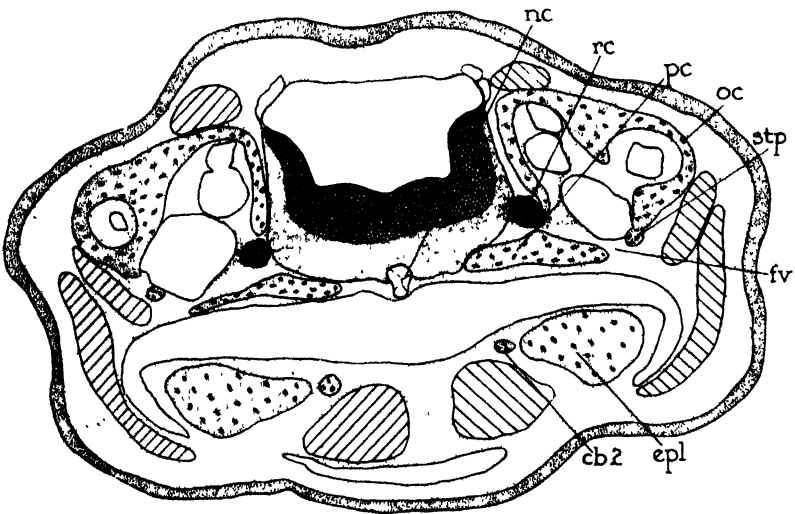


Fig. 5 Cross-section through the middle of the otic capsule (*oc*). The stapedial plate (*stp*) of the right side shows a direct continuation of the capsular wall, while that of the left has been sectioned at a level slightly posterior to this attachment. The ganglion of the ramus cochlearis (*rc*) is encased in its foramen. Ventrally, the first epibranchial (*ep1*) and the second ceratobranchial (*cb2*) cartilages are seen.

of these (fig. 6) are in the inner wall of the capsule. Three of the foramina of the inner wall are arranged in a plane parallel to the notochord, in the ventral part of the wall. The fourth, which marks the midpoint of the capsule, is dorsal and slightly posterior to the second of the three lower foramina. The first two of the lower foramina are equal in size and much larger than the third and fourth. The anterior (*x1*) of the lower three encloses the acusticofacialis ganglion, and

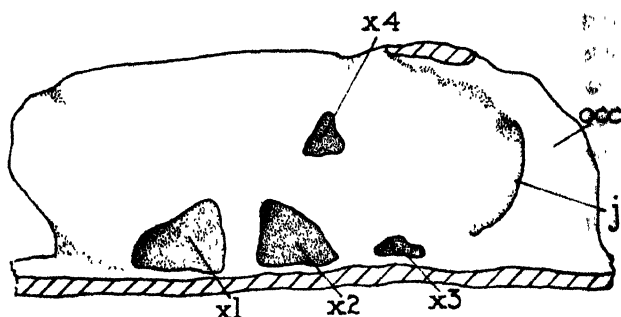


Fig. 6 Drawing showing the arrangement of the foramina of the medial wall of the otic capsule. The anterior one (*x1*) of the lower three foramina encloses the acousticofacialis ganglion; the second (*x2*) encases the ramus cochlearis of the auditory nerve; the third (*x3*) permits the ductus perilymphaticus to pass under the brain, while the fourth or upper foramen (*x4*) gives passage to the ductus endolymphaticus over the brain. The occipital arch (*occ*) and the foramen jugulare (*j*) are shown in relation to the medial posterior part of the otic capsule.

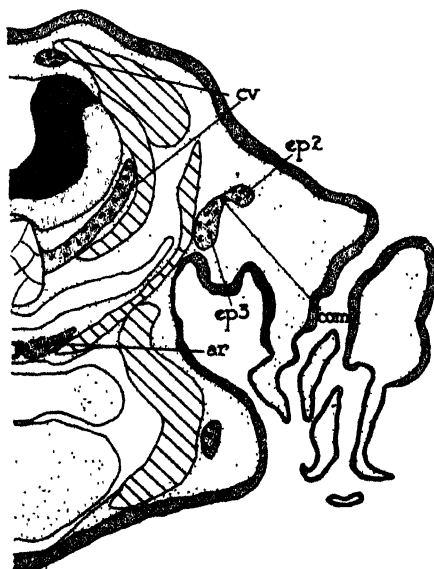


Fig. 7 Cross-section through the precartilaginous masses of the arytenoid (*ar*). At this point, the first cervical arch (*cv*) articulates with the notochord. Laterally, the second (*ep2*) and third (*ep3*) epibranchials, and the common terminal (*com*) joining them, can be seen.

from this nerves go through the adjacent cartilages. Two of the roots pass through two small foramina of the floor, ventral to the foramen of the inner wall. The second foramen (*x2*), which is separated from the first by a narrow strip of cartilage, gives passage to the ramus cochlearis of the auditory nerve, to the auditory vesicle. The third (*x3*) permits the ductus perilymphaticus to pass under the brain, while the

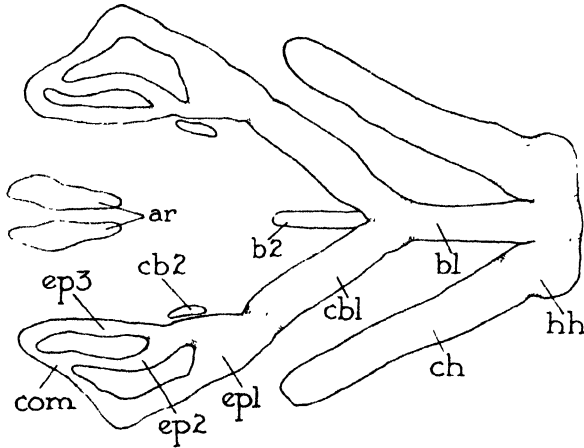


Fig. 8 Diagram showing the component parts of four of the visceral arches present in the 23 mm. larva of *Necturus*. The first visceral arch is not represented in this diagram. The arches shown are as follows: II) hypophyials (*hh*) and the ceratohyals (*ch*); III) first ceratobranchials (*cb1*) and epi-branchials (*ep1*, 2, and 3); IV) second ceratobranchials (*cb2*); and, VII) arytenoid cartilages (*ar*). The second and third arches are connected by the first basibranchial (*b1*). Although the connection between the third and fourth arches is incomplete, it is represented by the second basibranchial (*b2*).

fourth or upper foramen (*x4*) gives passage to the ductus endolymphaticus above the brain.

In the floor of the otic capsule are three foramina. The two anterior and smaller openings give passage to the branches of the seventh nerve. The third and largest foramen, the fenestra vestibule (fig. 5 and 9b, *fv*), is in the center of the floor. Its long axis marks the line at which the floor begins to turn slightly up to meet the dorsolateral side of the capsule. Lying in the anterior half of this foramen is a

rod-like cartilage, the stapedial plate (*stp*). The anterior portion of it is continuous with the floor of the otic capsule. The entire structure is within the plane of the floor.



Fig. 9, a and b Photographs of a model reconstructed from sections through the chondriocranium of a 23-mm. *Necturus* larva. A, dorsal view. B, lateral view. Neurocranium: *aop*, antorbital process; *bp*, basal plate; *cc*, column ethmoidalis; *cv*, first cervical arch; *fo*, foramen opticus; *foc*, foramen oculomotorius; *fv*, fenestra vestibule; *nc*, notochord; *occ*, occipital arch; *pb*, planum basale; *pc*, parachordal; *spl*, sphenolateralis; *stp*, stapedial plate; *t*, trabecula. Splanchnocranium: *ar*, arytenoid; *b*, first basibranchial; *b2*, second basibranchial; *ch*, ceratohyal; *cb*, first ceratobranchial; *cpl*, 2, 3, epibranchial; *hh*, hypohyal; *m*, Meckel's cartilage; *q*, quadrate; *pal*, palatobasale; *op*, otic process.

The occipital arch (fig. 9a, *occ*) is posterior to the otic capsule. Its basal margin is continuous with the posterior end of the basal plate. The arch, however, does not form a continuous floor for the neural tube at this point, as it is interrupted by the notochord. Each of its lateral processes extends outward from the notochord for a short distance. From the anterior part of the arch, each process continues dorsally, its outer side fusing with the posterior inner side of the otic capsule. Thus, the foramen jugulare, which encases the ganglionic mass of the glossopharyngeal and the vagus nerves, is formed between the arch and the capsule. The ascending process continues forward, along the dorsal part of the inner wall. After a short distance, a fourth of the length of the capsule, the two processes of the occipital arch converge abruptly, fusing to form a roof over the brain at this point. From here the arch extends forward, ending at the median point of the otic capsule.

The anterior margin of the base of the first cervical arch (figs. 7 and 9, *cv*) articulates with the posterior basal margin of the occipital arch. Both arches are formed in a quite similar manner. Each lateral process of this arch extends outward from the notochord for a short distance, continuing upward, and bending over the neural tube to meet and fuse with the other process of the arch. The formation of these two arches differ in that the dorsal plate, formed by the union of the two lateral processes of the first cervical, bends posteriorly rather than anteriorly as does the dorsal plate of the occipital arch.

The splanchnocranium (fig. 8) consists almost entirely of rod-shaped cartilages, extending the same distance forward and backward as does the neurocranium. A very marked resemblance to the primitive condition, as represented by the visceral arches, can be noted. Derivatives of these exist in the same plane and in the same relative positions, with the exception of the sixth, which is not represented in this larva. The first arch exists as Meckel's cartilage and the quadrate, the second as the hypohyals and ceratohyals, the third as the

first ceratobranchials and first epibranchials, the fourth as the second ceratobranchials and second epibranchials, the fifth as the third epibranchials, and the seventh is represented by two precartilaginous masses which will later become the arytenoid cartilages.

The two quadrate cartilages (fig. 9, *q*) furnish the points of connection between the neurocranium and the splanchnocranium. Each is a wedge-shaped structure consisting of two chief elements, the ascending and descending processes. The ascending process corresponds to the apex of the wedge. Its dorsal extremity fuses with the ventral side of the sphenolateralis of the neurocranium. From this point it extends lateroventrally, and as it widens merges into the descending process of the quadrate. The descending process, which corresponds to the base of the wedge, extends slightly forward to articulate with Meckel's cartilage. Posteriorly, it gives off two processes. One, the otic process (figs. 4 and 9, *op*), is a direct continuation of it, and the other, the palatobasale (figs. 4 and 9, *pal*), extends backward as a lateroventral process. Both coalesce with the anterior portion of the otic capsule, the otic process with its outer side and the palatobasale with its inner side. Thus, a foramen is formed between the two processes and the capsule which gives passage to one of the blood vessels of the head.

Meckel's cartilage (figs. 2, 3, and 9, *m*) extends forward from the quadrates as two processes which gradually converge and fuse with each other at their anterior extremities. Thus, an arch supporting the lower jaw is formed which extends forward, bridging the two quadrate cartilages. Each process at its point of articulation with the quadrate is a rather thick rod. Toward the anterior end of the head its diameter decreases, so that it has the shape of an elongated cone.

At a point opposite the center of Meckel's cartilage is a mass of precartilage. It is possible to distinguish three structures arising from this mass. The median one is the first basibranchial cartilage (figs. 3 and 8, *b1*) and the two lateral

are the hypohyals (*hh*). The first basibranchial continues back to the point of fusion of the two first ceratobranchials. The hypohyals extend posteriorly a short distance, and then coalescence with the ceratohyal cartilages.

The two ceratohyal cartilages (figs. 4 and 8, *ch*) diverge from the basibranchial, beginning at their point of fusion with the hypohyals. This is continued posteriorly until they end in the region back of the small foramina of the seventh nerve.

The two first ceratobranchial cartilages (figs. 4 and 8, *cb1*) fuse with each other at their anterior ends, as well as with the posterior end of the first basibranchial and the anterior end of the second basibranchial (*b2*). From this common point of junction of the four cartilages, the first ceratobranchials diverge in a posterolateral direction which continues to a point opposite the posterior ends of the ceratohyals, where each articulates with one of the first epibranchial cartilages.

The second ceratobranchial cartilages (figs. 5 and 8, *cb2*) lie slightly posterior to the point of articulation of the first ceratobranchials and the first epibranchials and extend along the inner side of the first epibranchials for a short distance. The second ceratobranchial cartilages are two short cartilaginous rods. They are not directly connected to the branchial skeleton.

The second basibranchial (fig. 4 and 8, *b2*), which is of a smaller diameter than the first, lies in the same median plane. It continues posteriorly from its point of fusion with the first ceratobranchials the same distance as the first ceratohyals, and ends as a free process in the surrounding connective tissue.

The two first epibranchial cartilages (figs. 5 and 8, *ep1*) continue the posterolateral divergence of the first ceratobranchials from the point of articulation of the two cartilages. Each of the first epibranchial cartilages becomes widened at its anterior extremity. Posteriorly from this widened end and from its inner side, the first epibranchial

articulates with the second epibranchial (figs. 7 and 8, *ep2*) which extends lateroposteriorly, parallel to the first epibranchial cartilage. The second epibranchial, near its anterior end, fuses with the anterior end of the third epibranchial cartilage (*ep3*), which also continues from the inner side of the second epibranchial in a lateroposterior direction. The first epibranchial does not extend posteriorly as far as the second and the second does not extend as far as the third. The posterior portion of the third epibranchial cartilage extends as far back as the arch of the first cervical vertebra. The distance between the first epibranchials at their posterior ends is the greatest lateral dimension of the chondrocranium. The three epibranchial cartilages at this stage are connected by a cross-bar at their posterior ends.

On each side of the anterior part of the trachea is a precartilaginous process. These two structures when chondrified will be the paired arytenoid cartilage (figs. 7 and 8, *ar*). This structure is a derivative of the fifth branchial arch. There is some evidence of chondrification at the center of each mass of precartilage. The general shape of the precartilage resembles that which exists at later stages of development. Each is of a triangular form, the base of the triangle being along the side of the trachea.

DISCUSSION

Although the skull of *Necturus* was described by Huxley ('74), Parker ('77), and, with reference to the hyoid and otic elements, by Cope ('89), the first accounts of larval chondrocrania known to us are those of Wilder ('95), who refers to a 25-mm. and a 44-mm. larva; of Platt ('96), who discusses conditions in larvae of 15, 19, and 46 mm., and of Winslow ('96), who used larvae of 21, 24, and 46 mm. The normal tables of Eycleshymer and Wilson appeared in 1909. Peeters ('10) gives an extended account of conditions in larvae of 20, 22, and 25 mm. Higgins ('20), in discussing the development of the nasal capsule, examined larvae of 24, 30, 33, 35, and 45 mm.

The widest differences of opinion are found with reference to the degree of chondrification of the sense capsules. In the larva here described the columna ethmoidalis is distinctly chondrified, a condition noted by Platt in the 46-mm. stage and by Higgins in stages later than 25 mm. His description of the columna ethmoidalis in a larva of 33 mm. corresponds quite well with that of our model.

The antorbital processes have centers of chondrification separate from those of the trabeculae. Winslow states that cartilage is formed prior to the 24-mm. stage, but Higgins denies this on the basis of his 24-mm. material. Our specimen agrees with that of Winslow in this respect.

Our specimen gives considerable support to the view of Wilder that the stapedia plate arises directly from the wall of the otic capsule. Platt and Kingsbury ('03, '08), on the other hand, consider that this structure arises independently of and external to the otic capsule. In the specimen here described the anterior end of the stapedia plate is a direct continuation of the wall of the capsule without the intervention of any precartilage, such as would be expected if an independent center of chondrification existed.

The bar described on the dorsal side of the trabecula, here called the sphenolateralis, a term apparently originating with Gaupp ('96) in describing the anuran chondrocranium, has heretofore been known as the crista trabeculae. Platt suggested the independent origin of this structure which is quite apparent in our material. The posterior prolongation of the sphenolateralis beyond the point where it fuses with the quadrate has not been observed before in larvae at this stage of development.

The planum basale is almost completely cartilaginous, although some precartilage is found between the plate and the trabeculae, giving evidence of its independent origin. Previous accounts either ignore this point or deny the formation of cartilage till a later stage (30 mm., Higgins).

This material lends no support to the view of Platt that a pro-occipital vertebra has been incorporated into the otic

capsules. The so-called tectum interoccipitale or synotic tectum is merely a continuation of the lateral processes of the occipital arch fused in the midline. This conclusion is in line with that of Peeters, although in his specimen of 22 mm. the lateral processes had not completely fused.

The palatobasale, first recognized by Peeters, is easily distinguishable in this specimen.

We are unable to distinguish any trace of chondrification in the fourth branchial (sixth visceral) arch of this specimen, although both Göppert ('95) and Wilder consider that part of the tracheal cartilages are derived from this arch.

SUMMARY

A description of the chondrocranium of a 23-mm. *Necturus* larva based on serial sections and a wax reconstruction indicates that the columna ethmoidalis is chondrified at this stage, that a cartilaginous antorbital process is present, and that the stapedial plate is cut out of the wall of the otic capsule. The so-called crista trabeculae is equivalent to the sphenolateralis of the *Anura*. The planum basale is cartilaginous. There is no evidence supporting the existence of a prooccipital vertebra. No trace of the fourth branchial arch could be found and the fifth exists only as precartilag.

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KEIMBAHN DETERMINANTS AND CONTINUITY OF THE GERM CELLS IN SPHAERIUM STRIATINUM (LAM.)

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TWO PLATES (EIGHTEEN FIGURES)

AUTHOR'S ABSTRACT

During oogenesis granular mitochondria become concentrated in the proximal portion of the oocyte and remain concentrated in the line of cells that gives rise to the germ cells. Two cells of characteristic structure, appearing at gastrulation one on either side in the mesoderm, contain this mitochondrial cloud. All functional germ cells seem to be lineal descendants of these two primordial germ cells. The cloud disappears during gonad formation, but reappears in oocytes of the next generation. Except during meiosis and fertilization the germ-cell cycle is traceable from fertilized egg to sexual maturity. Details of meiosis and chromosome number were not ascertained.

Capacity for producing germ cells in *Sphaerium* is traceable from a definite region in the egg to primordial germ cells through a localized cloud of mitochondria in the mature ovum and certain cells of cleavage stages. Mitochondria, therefore, serve as a Keimbahn determinant in the sense that they mark a region of oocyte cytoplasm destined for cytoplasm of primordial germ cells. They are not considered causal factors in production of germ cells, but are probably storage products persisting unused in the egg and in cleavage cells having lower metabolic rates. Disappearance of these mitochondria during gonad formation is explained by their dispersal among a number of cells and by their utilization in furnishing energy and material for production of new cells.

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INTRODUCTION

During an earlier investigation of the history of the germ cells in *Sphaerium* (Woods, '31) it became evident that certain cells of the cleavage stages contained granules that were lacking in others. It seemed that these granules occurred

only in those cells that later gave rise to the germ cells. This is not strictly true, since similar granules occur in all the cleavage cells but to a very limited extent. The work recorded in this paper is the determination by cytological methods of the nature, origin, distribution during development, and fate of these granules.

An attempt was also made to determine the details of meiosis and the number of chromosomes with a view to ascertaining whether certain of the primordial germ cells were destined to give rise to ova and others to spermatozoa, since there is no apparent difference in the primordial germ cells of the ovary and testis.

A review of the extensive literature concerning germ-cell continuity is unnecessary. This has been done a number of times as by Hegner ('14), Hargitt ('25 and '26), and others. A variety of forms can be cited in which germ-cell continuity has been described and in which certain structures in the egg are later associated with the germ cells. These germ-cell determinants, or, more accurately, 'Keimbahn' determinants, afford a means of tracing the capacity to produce germ cells through successive cell generations.

Metschnikoff ('65 and '66) first described the germ line in the paedogenetic larvae of the fly *Miastor*. Various investigators have demonstrated a complete 'Keimbahn' in other species. Hegner ('14) assembled the available information on the subject previous to 1914. Representatives from the coelenterates, nematodes, arthropods, and vertebrates are included as showing a traceable Keimbahn. Since this summary by Hegner, which dealt chiefly with the Keimbahn in insects, numerous papers have dealt with the subject in other groups (compare Goldsmith ('28), Heys ('31), and others). Hargitt ('25) in summarizing the evidence for and against germinal continuity in the vertebrates finds no evidence for early segregation of germ cells or for production of functional germ cells from the so-called primordial germ cells of the embryonic stages. This disagrees with the accounts of Foley ('27), who describes continuity of germ cells in the

teleost Umbra, of Rauh ('26) for the rat, Goldsmith ('28) for the domestic fowl, and Richards and Thompson ('21) for *Fundulus*. The more recent work of Heys ('31) summarizes the available literature on both sides of the question and presents evidence for continuity of germ cells in the rat on the basis of regeneration experiments.

It is evident, therefore, that at present no general statement concerning germinal continuity can be made that will hold true for all animal groups. Observations which throw light on the question of continuity or discontinuity of germinal material in any animal should be of value. This account of germ-cell determinants in *Sphaerium* furnishes means of demonstrating germinal continuity in this species in addition to that found in my previous paper on cleavage (Woods, '31).

MATERIAL AND METHODS

Specimens collected in the vicinity of Columbia, Missouri, and identified as *Sphaerium striatinum* were used in this work. This identification was verified by Mr. Byrant Walker, Honorary Curator of the Department of Molluscs of the Museum of Zoölogy, University of Michigan. Classification of material collected at Woods Hole, Massachusetts, was verified by W. J. Clench, Curator of Molluscs, Museum of Comparative Zoölogy, Harvard.

The general features of anatomy, development, gonad origin, and nutrition of the embryos have been described. The literature on these subjects has already been reviewed (Woods, '31). The animals are hermaphroditic. Eggs develop into miniature adults in brood pouches arising from the gill lamellae. Eggs and sperm are matured continuously throughout the year; and while experimental proof of self-fertilization is lacking, the disposition of parts is such that self-fertilization seems probable.

Specimens collected from submerged water plants or washed from the mud from the bottom of ponds were kept in tap water or clear pond water in the laboratory long enough for the discharge of hard materials from the digestive tract before fixation.

1. Fixation

Whole specimens, gills containing embryos in the brood pouches, and embryos dissected from the brood pouches under a binocular microscope were fixed according to the methods described in standard text-books of histological technique, for the following solutions: Allen's, Benda's, Bouin's, Bouin's with formic acid instead of acetic acid, Carnoy's, Carnoy's with formic acid instead of acetic acid, the fluid of Carnoy and Lebrun, Carothers' modification of Bouin's fluid, corrosive sublimate-acetic acid, corrosive sublimate-formic acid, Champy's, Flemming's, Guthrie's 'A' fluid consisting of 15.0 cc. saturated aqueous solution of picric acid, 1.0 cc. of 3 per cent chromic acid, 1.0 cc. of formic acid; Helly's, Zenker's, Zenker's with formic acid instead of acetic acid, Zenker's with copper sulphate instead of sodium sulphate and with the acetic acid replaced by formic acid. In addition to the better-known fixing fluids listed above, some of which are described in detail by Guthrie ('25), a method used by Guthrie (Guthrie 'B') consisting of one- to three-day fixation in a solution of 8.0 cc. of 5 per cent chromic acid, 8.0 cc. of 3 per cent potassium bichromate, 3.0 cc. of 2 per cent osmic acid, and 1.0 cc. of formic acid was found satisfactory. Benda's, Champy's, and Guthrie 'B' fixations were in some cases postchromed for twenty-four hours with a mixture of one part pyroligneous acid rectified with two parts 1 per cent chromic acid followed, after washing one-half hour in distilled water, by twenty-four hours' treatment with 3 per cent potassium bichromate.

Some of the material fixed in Helly's fluid was mordanted in Muller's fluid. Following fixation the material was dehydrated and embedded in paraffin. Sections were cut 4, 5, 7, and 8 μ .

2. Staining

A. For observations on the cytoplasm the following stains were used: Anilin-fuchsin-thionin-aurantia method of Kull, crystal-violet method of Benda, iron-alum-haematoxylin method of Heidenhain, iron-alum-haematoxylin method of Regaud, and the scharlach R method.

The first of these four methods was used most extensively because of its relative simplicity and the fact that it demonstrated mitochondria clearly.

Intra-vitam staining with Janus green, in specimens teased apart or in smears, permitted the demonstration of mitochondria in living cells.

B. For observations on the nucleus the following stains were used: Heidenhain's iron-alum-haematoxylin, Regaud's haematoxylin, Ehrlich's acid haematoxylin, Mayer's haemalum with orange G or eosin as a counterstain, toluidin blue and orange G, fuchsin and picro-indigo-carmin as described by Shumway (Stain Technology, vol. 1, p. 37). The rapid method of using Heidenhain's and Regaud's haematoxylin by flooding the slide with stain and heating until blue gave results equally as good as the longer method. The Feulgen reaction was also used following several methods of fixation. The original plan was to try all the possible combinations of the staining and fixing fluids listed and many of these combinations were thus tested, but only the more promising were fully utilized.

The method of making models of the early stages in development has been described, along with the manner of following particular cells through the cleavage stages (Woods, '31).

OBSERVATIONS

1. Origin and distribution of mitochondria in the ovarian egg

The general stages of growth in the oocyte of *Sphaerium* have been described by Stauffacher ('93). Application of cytological methods has made it possible to follow the granules previously observed from the time of their appearance in the oocyte to their disappearance from the primordial germ cells after they reach the developing gonad in the embryo (compare figs. 2 and 17).

When growth begins, the oocyte is distinguishable from the epithelial cells of the ovary by its large, spherical nucleus containing one or more large, deeply staining nucleoli (fig. 1). The nuclei of the epithelial cells are oval and smaller than

those of oocytes. The cytoplasm of the oocyte is more coarsely granular than that of the epithelial cells and there is also a difference in the ratio of nuclear and cytoplasmic volume in the two kinds of cells. Nuclei of young oocytes are actually and relatively larger than the nuclei of epithelial cells. Very young oogonia appear in every respect like primordial germ cells of later embryonic stages (compare fig. 1 with Woods, '31, figs. 37 to 46). While the material does not permit description of the details of meiosis, it does show that it is occurring when growth of the oocyte begins.

As growth progresses both nucleus and cytoplasm increase in size due to the addition of materials in two ways. The first of these is by absorption of material from adjacent cells in the wall of the ovary and from nearby vascular spaces. This process probably occurs during the entire growth period and is the only method by which materials for the synthesis of storage products are added during the early stages of growth. During the latter part of growth, just before the ovum is set free, this method of addition of materials is again the only one in operation.

The second method by which material is added to the cytoplasm of the growing oocyte is by an incorporation of whole cells or parts of epithelial cells from the wall of the ovary. There is no indication whether this is accomplished by ingestion on the part of the oocyte or by the activities of the epithelial cells themselves (figs. 4 to 7). In either case growth is well under way and the oocyte projects slightly from the wall of the ovary before any contribution of epithelial cells occurs (fig. 4). The growing oocyte projects into the cavity of the ovary with its basal end surrounded by elongated epithelial cells (figs. 4 and 6). In the early stages these cells lie with one side in contact with the oocyte. The membrane between the oocyte and an epithelial cell breaks down, the cytoplasm of the two merges and it is then impossible to determine which portion of the cytoplasm was a part of the original oocyte and which was contributed by the epithelial cell. The nucleus of the epithelial cell maintains its identity for a some-

what longer period. The nuclear membrane breaks down and for a time the chromatin granules can be seen in the cytoplasm (figs. 5 and 6). The disintegration of these chromatin granules marks the final disappearance of that particular epithelial cell into the cytoplasm of the oocyte. Many epithelial cells thus contribute to the formation of one oocyte.

In some cases only part of the cytoplasm of an epithelial cell enters the oocyte. In elongated cells with only the end toward the cavity touching the oocyte, the part in contact becomes detached from the basal part and is incorporated into the oocyte. The nucleus and the remainder of the cytoplasm of the epithelial cell maintain their identity (fig. 7).

The fusion of two growing oocytes to produce one has not been observed in any of my material, though Stauffacher ('93, fig. 10c) shows such a case.

Granular mitochondria are present in all cells of the gonad as in other cells of the organism. This agrees with the statement of Cowdry ('24) that all cells so far observed show mitochondria. Gatenby ('17) distinguished between oogonia and spermatogonia in the ovotestis of *Helix* on the basis of the distribution of mitochondria. Such a distinction is not possible in *Sphaerium*. There is, however, a difference in the distribution of mitochondria within the two types of cells constituting the wall of the ovary. Epithelial cells, which serve as nutritive cells if they happen to be adjacent to a developing oocyte, have a few mitochondria distributed throughout the cytoplasm without definite order. Very young oocytes on the other hand show a close condensation of mitochondria about the nucleus with a few scattered, peripherally located mitochondrial granules (figs. 1 and 2). It is not necessary for the present work to propose theories to account for this location of mitochondria in oocytes which are just entering the growth period, although the problem is important (compare Guthrie, '25). Similar condensations about the nucleus have been described by N. H. Cowdry ('17) in meristem tissues in plants, Regaud ('08 and '10) during spermatogenesis in the mouse, Tsukaguchi ('14) in ovarian eggs in

Aurelia, the Lewises ('15) in tissue cultures, and by other investigators.

As growth of the oocyte continues there is a great increase in the number of mitochondria and a change in the location of the mitochondrial cloud. Immediately preceding and during the early part of the period in which nutritive cells become a part of the oocyte, the mitochondria are found at the proximal end of the oocyte (figs. 3 to 7). In this location they were adjacent to the region of the cell where materials are entering from the circulatory system. Similar location of mitochondria has been described in kidney cells, cells of the intestinal mucosa, thyroid gland, oocyte of the frog, etc.

This proximal mass of mitochondria persists throughout the period during which the oocyte is attached to the wall of the ovary. During the contribution of materials by the nutritive cells there appears a continuous cone of mitochondria extending from the proximal end of the cell up to and surrounding the proximal side of the nucleus (figs. 6 and 7). A few scattered mitochondrial granules always occur throughout the cytoplasm. Gatenby ('19) in *Helix* and Rauh ('26) in the rat distinguish two kinds of mitochondria on the basis of size. In *Sphaerium* difference in size does not seem significant because various gradations in size occur throughout the cytoplasm.

Nothing definite can be said concerning migration of mitochondria, since living ovaries have not been studied. However, just before the ovum is set free from the wall of the ovary the perinuclear cloud of mitochondria disappears and is replaced by a clear area which stains faintly pink with acid fuchsin. The mitochondrial cloud at the basal end persists and becomes denser at this time, possibly indicating movement of the perinuclear mitochondria into this region. A different localization of mitochondria in the full-grown attached eggs and eggs free in the ovary also seems to indicate migration of the mitochondria. In attached eggs the mitochondria are symmetrically distributed about the proximal end (fig. 7). In the egg free in the ovary the main mass

of mitochondria is slightly to one side of the micropyle which marks the point of previous attachment (fig. 8). A rotation of the egg within the egg membrane would also account for this latter change in location of the mitochondria. Fixed material gives no proof of which is the case. When the egg starts down the hermaphroditic duct the mitochondria are chiefly condensed in a cup-shaped cloud of various-sized granules to one side of the micropyle and about one-fourth the distance from the egg membrane to the nuclear membrane. Cleavage later demonstrates that this is in the region of the vegetative hemisphere (figs. 8 and 9). The few other mitochondria are irregularly distributed throughout the cytoplasm.

2. Distribution during cleavage and the period of inactivity of the germ cells

The situation in the mature egg just described results in the cloud remaining in the larger of the two cells resulting from the first cleavage (fig. 10). This cell is designated as *CD*, the smaller cell as *AB*. Division of *AB* to produce *A* and *B*, which constitutes part of the second cleavage, does not affect the location of mitochondria in *CD*. The spindle of the other second cleavage, by which *CD* is separated into its component cells *C* and *D*, is tilted so that *C* is given off adjacent to *A* and *B* on the upper surface of *D* (fig. 11, and Woods, '31, fig. 8). Hence the cell *C* receives very little or none of the mitochondrial mass, because the mitochondria lie about the lower end of the spindle in the region of the future cell *D*. Thus a four-cell stage is reached in which a few scattered mitochondria appear in each cell, while cell *D* shows a cloud of mitochondria of essentially the same size as in the mature egg. As previously shown (Woods, '31), the spiral type of cleavage which produces the successive generations of micromeres from the cell *D* is such that the first three micromeres are cut off from the upper surface of *D* and receive none of the mitochondrial cloud. The next division in *D* to produce a fourth micromere, *d.4*, is approximately

equal. The cleavage plane is almost at right angles to the first cleavage plane in *D* so that the mitochondrial mass passes mostly into *d.4*, although a small part of it remains in *D*. During the alternate rotation of the spindles producing the first three generations of micromeres the cloud of mitochondria either becomes shifted within the lower part of the cell *D*, or the entire cell changes its position. Though the cause of this changed position is not known, the result is that the mesoderm-forming cell *d.4* receives the main mass, while the endoderm-forming cell *D* receives only a small part of the rim from the cup-shaped mitochondrial cloud (Woods, '31, fig. 14).

With the equal division of *d.4* or *M* (mesoderm-forming cell) at right angles to the direction of the plane which separated *d.4* from *D*, the mass of mitochondrial granules is divided equally.

As the two mesoderm-forming cells sink into the blastula cavity each buds off three generations of small mesoderm cells into the cavity in such a way that at the end of the process these small cells and their descendants lie between the large cell and the body wall on either side. The mitochondrial cloud remains in the large cell (fig. 14; also Woods, '31, fig. 32). Mitochondria occur in the small cells, but to a very limited extent. They seem to be the scattered mitochondria and not to be derived from the main mitochondrial mass. Each of these large cells, which are the primordial germ cells, divides once and equally, resulting in each cell receiving apparently an equal portion of the mitochondria. These two primordial germ cells on either side of the embryo (Woods, '31, figs. 33 and 34) now enter a relatively long period of inactivity, which continues until they have reached their position in the definitive gonad.

3. Disappearance during gonad formation

After the four primordial germ cells have reached their final position they increase in number and development of the gonad begins. During this passive change in location

each cell contains a small cloud of mitochondria around the nucleus (fig. 15).

The succession of cell divisions, which results in an indefinite number of primordial germ cells in the gonad, divides the mass of mitochondria so that it is distributed about equally to the resulting cells. During these divisions a gradual disappearance of the mitochondrial cloud occurs and only a few scattered granules are present after the first few divisions (compare figs. 15 to 17). After this the primordial germ cells are comparable in appearance with those of the adult gonad (fig. 18; also Woods, '31, figs. 37 to 46 and fig. 1).

No methods so far have demonstrated the details of meiosis. The chromosomes are very small and appear as indistinct darker masses in a more faintly staining mass and are always clumped on the spindle. It was hoped that there might be visible differences in the primordial germ cells that would forecast which would become spermatozoa and which ova, but no such differences are apparent. Oogonia and spermatogonia are indistinguishable except for their difference in location. The cytoplasmic changes in spermiogenesis are fairly clear and it is hoped that methods may be devised to show the nuclear changes, thus permitting a complete description of spermatogenesis.

DISCUSSION

A general discussion or review of the literature on mitochondria is unnecessary for this work. Such reviews have been made by Meves ('15), Cowdry ('24), and others. It seems that cytological methods furnish reasonably accurate means of identifying mitochondria in fixed material. The question of mitochondrial continuity (Cowdry, '24) or their chemical composition (Mayer, Rathery, and Schaeffer, '14) have no direct bearing on the present work. I am concerned with the function of mitochondria only so far as function may explain their persistence in certain cells. Mitochondria have at various times been held responsible for the presence of practically all sorts of cytoplasmic inclusions and for dif-

ferentiated regions of the cytoplasm. I am concerned with mitochondria as visible structures present in such a way that they serve to mark a particular line of cells during cleavage. Two other investigators have described mitochondria as 'Keimbahn' determinants. Rubaschkin ('10 and '12) and Tschaschin ('10) find that granular mitochondria in the cleavage stages are replaced by chains and threads in differentiating somatic cells, while granular mitochondria persist in primordial germ cells. von Berenberg-Gossler ('14), the Lewises ('15 and '24), and others have shown that germ cells do not contain a specific kind of mitochondria. Montgomery ('11 and '12) considers mitochondria as causal factors in cell differentiation and states ('11, p. 791), "any cell of the cleavage stages that failed to receive mitochondria, or failed to receive particular ones or a particular amount of them would be incapacitated from engendering such somatic specialization (fibrillar structures), it would thereby become a germcell." Montgomery's hypothesis that the mitochondria of prospective germ cells remain unaltered or latent, while those of other cells undergo developmental changes, is interesting if granular mitochondria can be considered as reserve nutritive substances. In *Sphaerium* mitochondria seem to be the only storage product in the egg.

Guthrie ('25) believes that during storage mitochondria are precipitated in the cytoplasmic region where the amount of incoming materials is greater than the amount being used in cell metabolism. It would follow that mitochondria persist because they are not used up. If this theory is correct, it is possible to explain why so many mitochondria persist through the germ-cell-producing line and in the primordial germ cells until they resume division in the developing gonad. That the lower pole of the egg is less active is shown by the fact that new cells are cut off from the animal pole and tend to divide more rapidly than the remaining portion. Mitochondria would, therefore, tend to persist in the less active region about the vegetative pole. It is this region that gives rise to the mesoderm with the primordial germ cells, and to the

endoderm. The passage of the mitochondrial cloud into the primordial germ cells instead of into other mesodermal cells can be explained on the same basis. Similar interpretations have been placed upon the persistence of nutritive substances in general in primordial germ cells by Wheeler ('99) and by Hegner ('14), who states that primordial germ cells remain in the primitive condition and do not undergo differentiation at the same time or at the same rate and hence do not at first utilize their yolk contents since their metabolic activities are so slight. He further states: "It is more difficult to answer why these nutritive substances are segregated in the primordial germcells." In *Sphaerium* this segregation of reserve nutritive substances may be interpreted as wholly a matter of the mechanical relations of cleavage and the retention of reserves by regions of the cytoplasm where metabolism is carried on at a relatively lower rate.

After the primordial germ cells have reached their place in the gonad, cell division involves the whole cell in a way that is different from the process in the cell divisions producing micromeres. Except for the production of *d.4*, micromere production involves chiefly the upper part of the cell and the lower part is apparently little affected. The primordial germ cells, on the other hand, divide equally. While the disappearance of the cloud of mitochondria may be partially explained as a matter of dispersal among a number of cells so that the number present in any one is less than when there were fewer cells, it may also be explained in another way: If the mitochondria are reserve substances, their decrease in number at this time is probably as much a result of their utilization to furnish the energy for cell division and material for the production of cytoplasm as of their being distributed among a large number of cells. If they are used in this fashion as reserve nutrients, they are never completely used up or new mitochondria are formed, since there is never an entire absence of mitochondria in the primordial germ cells.

Although the history of cytology justifies the suspicions often held regarding bodies revealed by fixed and stained material, there is no doubt that many, if not all, of the cytoplasmic inclusions that have been designated as 'Keimbahn' determinants are realities and not artifacts. Some have been seen in living eggs; many have been described by several investigators; many are constant after being fixed and stained in several different solutions. Nucleoli, chromatin, chromidia, yolk, nurse cells, yolk nuclei, mitochondria, metabolic products, and various other structures to which special names have been applied have been designated as associated with the germ line in animals. Hegner ('14) discusses each of these determinants (pp. 460-478). Hargitt ('25) and others have discussed criteria for the recognition of germ cells. In most cases Keimbahn determinants are not considered the causal factors in the production of germ cells, but rather the results of mechanical and metabolic factors in the development. The ground cytoplasm may have localized in it during maturation, fertilization, and cleavage visible structures that mark the region or regions of the egg from which certain parts will arise in later development. Cytoplasmic movements and differences in the rate of metabolism in different regions of the cell are probably responsible for this localization.

The distribution of the mitochondrial cloud during cleavage and gastrulation in *Sphaerium* provides a means of identifying a particular region of the egg cytoplasm, which later becomes part of the primordial germ cells. These mitochondria can be designated as 'Keimbahn' determinants in the sense that their presence as a localized cloud in a cell during cleavage indicates that this cell will give rise to primordial germ cells just before gastrulation.

Mitochondria are not in any sense to be considered causal factors in production of the primordial germ cells. They serve as markers of the path along which is transmitted during the cleavage stages, the capacity to produce cells recognizable as primordial germ cells in this species.

The more general aspects of germinal continuity and early segregation have been discussed previously (Woods, '31, and Heys, '31). While germinal continuity has been maintained for some species and disclaimed for others, it is evident in *Sphaerium* that a definitely traceable line of cells gives rise to primordial germ cells from which all functional germ cells arise. There is no evidence that somatic cells give rise to germ cells.

SUMMARY AND CONCLUSIONS

In *Sphaerium* the capacity for producing germ cells is traceable from fertilized egg to the primordial germ cells of the developing gonad by means of a localized cloud of mitochondria in certain cells of the cleavage stages.

The origin and localization of these mitochondria in the developing egg are described, without attempting to determine the causes for their apparent shifts in position.

The cycle of the mitochondrial cloud, which is the Keimbahn determinant in this species, may be summarized as follows:

1. Appearance and change in location in the developing oocyte.
2. Localization in the cell *D* of the four-cell stage and during the production of the first three generations of micromeres.
3. Passage into the fourth micromere produced from the cell *D* and thence into the mesoderm-producing cell on either side during later cleavage stages, and finally into the primordial germ cells, which are the remaining cells one on either side, after the production of three generations of smaller mesoderm cells.
4. Absence of the mitochondrial cloud in early oogonia.

5. Reappearance in the oocyte during the growth period.

This mitochondrial cloud is not considered in any sense a causal factor in the production of primordial germ cells, but is held to represent reserve nutrients that persist in cells and in regions of cells having lower rates of metabolism,

hence their reserves are not used up for a long period during which differentiation proceeds in other regions. Its segregation into the line of cells from which germ cells arise is a result of the mechanical factors of cleavage.

The details of maturation and the chromosomal number could not be determined, although several fixing and staining methods were tried.

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DESCRIPTION OF PLATES

Figures 1 to 7 are outline drawings made from photographs of more detailed camera-lucida drawings after the method described by Naylor ('31). Figures 8 to 18 are camera-lucida drawings. No cytoplasmic structures except the mitochondria are shown. The magnification after reduction incident to reproduction is indicated for each figure.

Figures 1 to 8 represent stages in growth of the oocyte.

Figures 9 to 18 represent embryonic stages from fertilized egg to embryos ready to be set free from the brood pouch.

ABBREVIATIONS

<i>A, B, C, D</i> , macromeres	<i>mic</i> , micropyle
<i>b w</i> , body wall	<i>mit</i> , the mitochondrial cloud which is the Keimbahn determinant
<i>c go</i> , cavity of the gonad	<i>n per</i> , nucleus of a peritoneal cell of the gonad
<i>c m</i> , egg membrane	<i>nuc</i> , nucleolus
<i>end</i> , endoderm	<i>per</i> , peritoneal cell of the gonad
<i>g c</i> , germ cell	<i>s c</i> , segmentation cavity
<i>h p n</i> , anlage of the heart, pericardium, and nephridium	

PLATE 1

EXPLANATION OF FIGURES

- 1 Young oocyte just entering the growth period. Mitochondrial cloud beginning to appear in a perinuclear position. $\times 688$.
- 2 Slightly older oocyte showing increase in perinuclear cloud. $\times 658$.
- 3 Oocyte at time of beginning formation of egg membrane. Mitochondrial cloud now in the proximal side of the oocyte. $\times 900$.
- 4 Oocyte at the beginning of period of incorporation of peritoneal cells into the cytoplasm. $\times 585$.
- 5 An older oocyte showing the remains of one of the nuclei of a peritoneal cell within the cytoplasm. There is some shrinkage in this cell. $\times 450$.
- 6 A later stage in the growth of the oocyte showing the cone of mitochondria extending from the proximal end toward the nucleus. $\times 450$.
- 7 A three-quarters-grown oocyte in longitudinal section. $\times 450$.
- 8 Longitudinal section of fully grown egg free in the cavity of the ovary. The mitochondrial cloud has shifted in position. $\times 500$.
- 9 Longitudinal section of a fertilized egg in the brood pouch. The nucleus is in the early prophase of the first cleavage as indicated by the characteristic lobulation and decreased staining capacity of the nucleolus. The egg membrane is not shown. $\times 450$.
- 10 Two-cell stage showing mitochondrial cloud in the cell *CD*. $\times 450$.

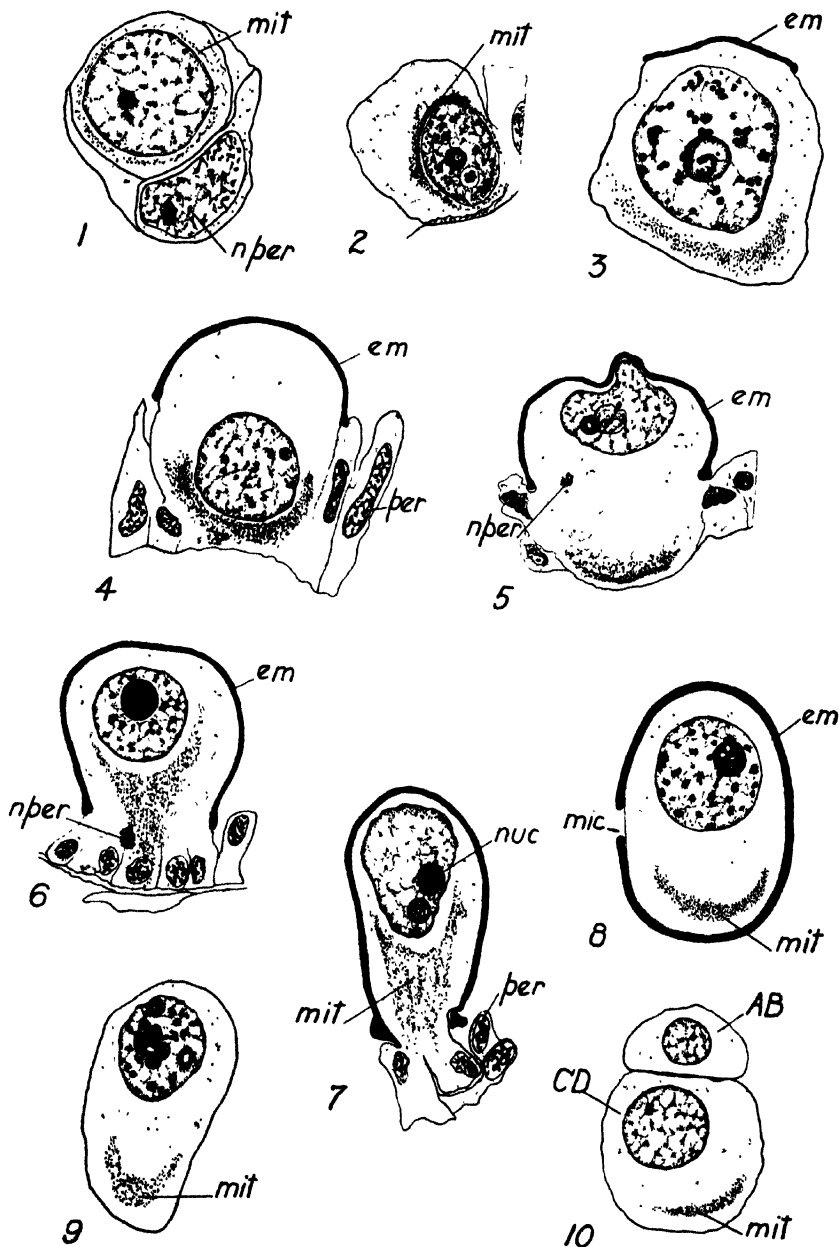


PLATE 2

EXPLANATION OF FIGURES

11 Section through a four-cell stage. The mitochondrial cloud is in the lower portion of cell *D*, while the other cells show only scattered mitochondria. $\times 450$.

12 Section through a nine-cell stage showing the cloud in the cell *D*. $\times 685$.

13 Section through a blastula of about twenty cells. No mitochondrial cloud appears in any cell except cell *D*. $\times 685$.

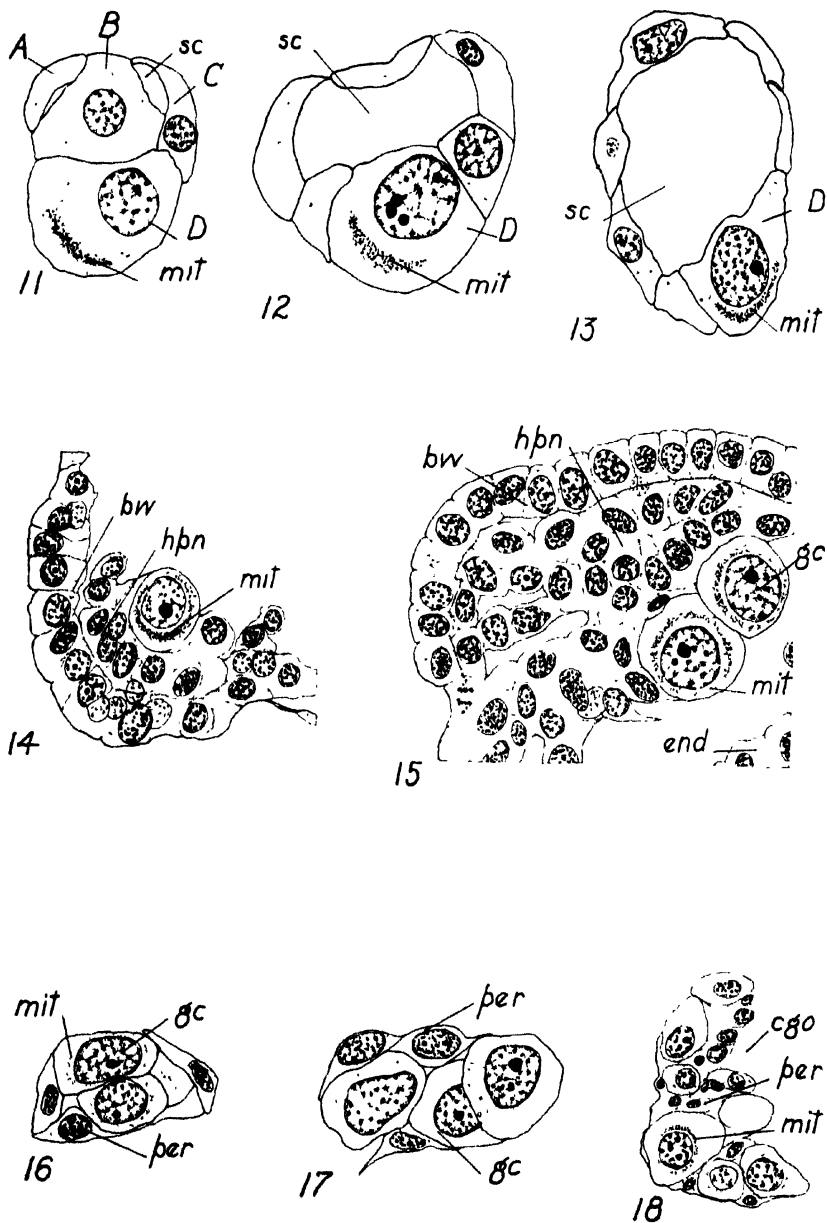
14 Lower left quarter of a section through an early gastrula showing the single primordial germ cell on the inner surface of the mesodermal mass. $\times 685$.

15 Section of an embryo at a stage in which the primordial germ cells have reached the place of gonadal formation, but have not yet resumed cell division. The persistent mitochondrial cloud is shown. $\times 935$.

16 Cross-section of the gonad at an early stage after the primordial germ cells have begun to divide. Only two of the six primordial germ cells appear in the section. $\times 685$.

17 Section through the developing gonad of an older embryo. Three of the twelve cells present show. The mitochondrial cloud has disappeared. $\times 935$.

18 A portion of a section of the gonad at the time the embryo is ready to be set free from the gonad. Certain oocytes are beginning growth and the mitochondrial cloud is reappearing in them. $\times 456$.



A MICROSCOPICAL STUDY OF GLOCHIDIAL IMMUNITY¹

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ONE PLATE (NINE FIGURES)

AUTHOR'S ABSTRACT

Glochidial cysts on the gills of immune fishes form in the same manner as normal ones, but they tend to grow larger and become more irregular. The increased thickness is due to additional cellular connective tissue in the wall. The gill tissue indicates the existing biological incompatibility only by the presence of eosinophiles, extruded chromatin spherules, and eosinophilic plastids.

In natural, or racial immunity many glochidia are promptly destroyed by cytotoxicity, accompanied by an invasion of host cells. These disintegrating glochidia may occur in close proximity to unaffected glochidia and apparently are merely less resistant individuals that succumb to a critically adjusted reaction.

In both natural and acquired immunity the normal retention of glochidia and the accompanying metamorphosis are replaced by premature shedding. After the first day, the cyst thins by the removal of stroma cells back into the filament until the wall is reduced to a thin envelope. Both intact and destroyed glochidia, and apparently their cyst coverings, are sloughed at about the second day. Repair of the resulting notched filament is prompt.

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INTRODUCTION

Within recent years it has been learned that a definite immunity is associated with the development of fresh-water mussels. This immunity is operative at a time when the larval glochidium normally becomes a temporary parasite on its fish host, and is expressed through the refusal of the fish to serve as host. The immunity may be either natural or

¹ Contribution no. 170. Published by permission of the United States Commissioner of Fisheries. Acknowledgment is due the staff of the Fairport Biological Station for many helpful courtesies extended during the prosecution of this inquiry.

acquired. In both instances glochidia attach and encyst in the superficial tissues of gill or fin, but are not retained longer than one to three days.² Underlying natural, or racial immunity is the fact that the various mussel species are usually restricted to one or more particular fish hosts. Fishes that are not hosts will receive and encyst the glochidia of any mussel that can attach, but will not retain them. On the contrary, acquired immunity is an individual reaction whereby natural hosts that have experienced two, three, or more infections³ become incapable of carrying subsequent ones through the metamorphic period.

As a part of a program which has included experimental and microscopic studies on free and encysted glochidia (Arey, '21, '23 a, '24, '32 a, '32 b, '32 c) there was included an investigation of immunity, both natural and acquired. The objectives sought were to determine the histological conditions in host and parasite during their brief association, together with the changes anteceding rejection. A preliminary communication of the conditions found has already been published (Arey, '23 b).

A clear instance of natural immunity is afforded by the yellow sand shell, *Lampsilis anodontoides*, whose parasitism is restricted to the gar pikes. Infections upon such a non-host as the large-mouth black bass, *Micropterus salmoides*, are not retained beyond the second day. A closely graded series was collected and studied both totally and in serial section. The seventy-eight gill cysts are distributed by age as follows:

<i>Age of cysts in hours</i>	<i>Number of cysts examined</i>	<i>Age of cysts in hours</i>	<i>Number of cysts examined</i>
9	7	36	2
15	16	39	12
21	9	40	4
24	13	42	6
31	9		

²This and all subsequent statements refer to conditions as found in infections at midsummer temperature at Fairport, Iowa.

³In some instances one or two infections produce acquired immunity, but such animals are large and doubtless have harbored natural infections before capture (Arey, '23).

For acquired immunity the fat mucket, *Lampsilis luteola*, on the gills of its natural host, the large-mouth black bass, was used. These bass had become immune at the third infection and had promptly sloughed the fourth attempt at encystment. The series collected came from the fifth infection, when it was certain the immunity was well established. Some supplementary material was taken from the fifth infection of another lot. Fresh and preserved gill filaments, total mounts, and sections were utilized. The serial sections were of forty-four complete cysts, timed as follows:⁴

<i>Age of cysts in hours</i>	<i>Number of cysts examined</i>	<i>Age of cysts in hours</i>	<i>Number of cysts examined</i>
4	2	33	4
9	7	47	9
21	6	50	6
27	2	67	6
30	2		

All gill filaments received Zenker fixation within a few seconds after removal from the fish. Sections 6 μ thick were stained in hematoxylin and eosin.

OBSERVATIONS

Gross formation of immune cysts

The manner of attachment and the process of encystment conform to the normal procedure described in detail elsewhere in these reports (Arey, '21, '24, '32 a, '32 b). Yet an important size difference soon becomes apparent. In the acquired immunity of the *Lampsilis luteola* series the glochidia are about half-covered over at thirty minutes, and at that time the growing cyst still appears normal. At two and one-quarter hours the glochidium is two-thirds enclosed, but sits in a crater with thick lips and broad, rolling edges. Shortly after four hours it becomes completely overgrown. These immune cysts tend to grow larger than normal ones and often are strikingly bulky (fig. 1). They also show less restraint and commonly become irregular, malformed tumors

⁴ Stages at fifty and sixty-seven hours were from the fifth infection material just mentioned.

with prominent irregularities and horizontally spreading wings. Toward the end of the first day they reach a maximum size and thereafter reduce in a manner to be described presently.

The same general history holds for the naturally immune *Lampsilis anodontoides* series. In both instances, the greater growth exuberance in immune stages over normal ones is manifest. As a whole, the cysts are notably larger and more irregular. It seems as though some regulatory principle effecting terminal growth ceases to operate. A comparable circumstance is found in the formation of cysts on the excised gill filaments of normal hosts (Arey, '21).

Histological changes in the host tissue

Structurally the early immune gill cyst is like the normal, but with thicker walls. This massiveness is due wholly to additional cellular connective tissue. In another publication (Arey, '32 b) it is shown that encystment results from an active migration of cells which cover the glochidium, and not from mitotic proliferation. All the immune material, procured and studied subsequently, has confirmed this earlier conclusion. Elsewhere (Arey, '32 a) the point has also been stressed that the gill cyst is in no sense an exclusively epithelial cyst, if indeed it can properly be called a cyst at all. The prevalent error of interpretation has resulted from confusing the epithelium with the cellular stroma beneath it. The two are usually poorly demarcated, and often appear confluent. Some of the present series show these boundaries with greater distinctness than has been observed before (compare fig. 1; right margin of cyst). The 'cyst,' then, at its height is a tumor-like mound of connective tissue in which the glochidium lies buried; externally is a mantle of epithelium continuous with the general gill covering (figs. 1 and 6).

As with the method of formation and general structure, so also in histological composition the immune cyst adheres closely to the normal. An interesting feature is the occurrence of eosinophiles. In the naturally immune *Lampsilis*

anodontoides series, scattering eosinophiles are found in the vicinity of many glochidia. These have been seen in the earliest stages examined (four hours), but do not occur in the normal bass gill.⁵ In the *Lampsilis luteola* sections eosinophiles are much more abundant, and are most plentiful in the younger stages. They tend to accumulate, at least as early as the four-hour stage, in the immediate vicinity of the glochidium, and frequently occupy a pocket or even a channel bordering the larva (fig. 1). In the later hours of encystment they are not as numerous, but occasionally may be seen scattered throughout the gill substance.

Both natural and acquired immunity lead to the production of eosinophilic plastids in the stroma. These are infrequent in the filaments infected with *Lampsilis anodontoides*, but can be found. About the *Lampsilis luteola* parasites they are commoner and occur at least as early as twenty-one hours.⁶ The origin of such elements is problematical.

Another characteristic component of the *Lampsilis anodontoides* material is a small, spherical, deeply basophilic mass. Many such may be seen in a single section. They appear to be extruded chromatin, for identical bodies occur in the nucleus, in the cytoplasm adherent to the nucleus, free in the cytoplasm and interspersed between stroma cells. Where such bodies are frequent the nuclei tend to be pale and poorly chromophilic; often this is strikingly true.

It would seem that the basophilic spherules, and probably the eosinophilic plastids and marshaled eosinophiles, are a visible expression of tissue incompatibilities associated with immunization. They represent the only morphological index of the subtle tissue reactions undoubtedly taking place in

⁵ A graded series of *Lampsilis anodontoides* on its natural host, the gar pike, did show some eosinophiles in the stroma of filaments. But this occurred only in certain filaments, all of which perhaps came from one or more of the six individuals used. It possibly represents a partial acquired immunity, for all the fish not only had received one previous infection, but their earlier experience as hosts while wild is unknown. One speculates on a possible correlation with the presence of fish lice which infest the gills of these animals.

⁶ No stages were available in my series between nine and twenty-one hours.

the host. We shall see presently that a deleterious effect is plainly discernible in the parasite.

Sometimes cyst-like vesicles, which may contain a cellular mass, occur. These are usually too small to have held a glochidium, and the absence of cuticular or other diagnostic remains (vide infra) furnishes definite disproof of such origin. In certain instances they communicate with true glochidial cysts or are attached like blebs to their peripheries. Of somewhat the same nature are tumorous masses which externally appear like true cysts, but prove to be made of solid gill tissue with at most a spongy cellular interior. Both presumably represent proliferations expressive of the reactive exuberance of the stimulated immune filament. This tendency to overgrowth is further illustrated by the size and irregularity of the true glochidial cysts. Not only are irregular outgrowths very common, but the cyst tissue as a whole often spreads outward along the filament for some distance.

Cytolytic destruction of glochidia

The attached glochidium may meet a prompt death, accompanied by the dissolution of its cellular components (figs. 2 to 4). This has been observed only in the *Lampsilis anodonoides* infection, which was utilized as a type to illustrate the natural immunity peculiar to non-hosts. In the *Lampsilis luteola* series, attached to its natural host which had acquired immunity by repeated infection, glochidial disintegration was not found. As the examination included representatives of two different *L. luteola* infections, it would appear not to be characteristic of acquired immunity in this mussel.⁷ Whether

⁷ In contrast should be noted Reuling's ('19) statement for the same host and parasite. He subjected *Lampsilis luteola* glochidia to the blood of normal black bass and to blood from animals that had acquired immunity. The normal control remained for forty-eight hours unchanged. After twelve hours the glochidia in the immune serum desquamated their mantle epithelium, which then broke up into cellular debris; in some cases the valves opened. Finally, the entire internal structure was destroyed. Also he reports that glochidia examined after twenty-four hours' attachment to the gills of immune bass "showed a certain amount of disintegration." It should be recorded that most of the *Lampsilis luteola* material used by me in this study came from Reuling's later infections. It is puzzling to account satisfactorily for the discrepancy in our results.

such a conclusion will apply as a generalization to all cases of acquired immunity cannot be predicted.

Not all of the *Lampsilis anodontoides* glochidia are destroyed by cytolysis. Many* remain intact until lost (figs. 2, at right, 5 and 6). Those that do succumb may show early disturbances—an advanced state of degeneration has been observed at fifteen hours (fig. 2, at left). During this decline the glochidial tissues become necrotic, fragment into groups, and are more or less dislocated from their natural positions. The end result is often a state of disarrangement, disorganization, and disintegration (figs. 2 to 4). The adductor fragments, and its fibers swell and stain feebly as they disappear. Representatives of all the glochidial structures are recognizable for a relatively long time, but eventually the only remnants that can be surely identified may be the cuticular components of the valves (fig. 4, *h*).

With the early death of a glochidium, and the consequent relaxation and disruption of the adductor (fig. 3), the elasticity of the hinge spreads the valves apart in many instances (figs. 2 to 4). This distends the cyst and changes its shape, so that in such a disorganized condition it usually simulates two communicating cysts. The bitten host tissue may then appear like a partial partition.

Coincident with glochidial necrosis goes an invasion of host cells in greater or smaller numbers (figs. 2 to 4). Some early stages show an attack from one side only. These invasive elements are apparently cells of the stroma, to which eosinophilic elements, and perhaps other leucocytes, may be added. It is difficult to identify special cell types in most instances, and hence origins are obscure. Although it is tempting to infer a sequential relationship between this invasion and the accompanying retrograde changes, such is not amenable to proof. The two may be parallel phenomena, or the invasion may be secondary to the incipient degeneration and induced by it.

* Fifty-one cysts, twenty-one hours or more in age, were examined. Of these, nineteen were necrotic.

An astonishing feature is that highly necrotic glochidia occur on the same filament and often in apposition with glochidial cysts that show no apparent regressive changes (fig. 2). Advantages of position or nidus are not responsible. The phenomenon must depend either on local differences in the intensity of the immune principle, or to a variability in glochidial vitality and resistance. The first alternative is difficult to reconcile with prevailing ideas as to the nature of immunity.

Unaffected glochidia of the later hours, before shedding occurs, sometimes show mitoses in the organ-anlage mass, thus indicating that, like the normal larva, they are capable of attempting differentiation. This start toward metamorphosis was illustrated by the larva sectioned obliquely in figure 5.

The sloughing of glochidia

The manner in which glochidia are finally shed demands consideration next. Those glochidia which are not destroyed internally, according to the method just described, are lost intact. Such loss occurs frequently in natural immunity and was the only method observed in the material illustrating acquired immunity.

In neither instance do the glochidia show distinctive signs of injury. The bitten host tissue undergoes in situ (extracellular) digestion in the usual manner (figs. 1 and 6). This is accompanied by the coincident loss⁹ of mantle zymogen granules and the ingestion of bits of host cells by the mantle.¹⁰ After the normal latent period, mitoses appear in the mass of cells from which the future organs develop; such dividing cells are found even to the time when the glochidium is about to be cast off.

Structurally, the course of events leading to sloughing of the glochidium is not spectacular. The period of the bulky, thick-walled cyst is followed by a phase of thinning, during

⁹ In *Lampsilis anodontoides* this was a diminutional rather than total loss.

¹⁰ These events are described fully in another contribution (Arey, '32 c).

which the glochidium approaches an uncovered condition. This reduction often progresses unevenly, so that one portion of the cyst is much thinner than the rest (figs. 7, *a*, and 8, *a*). In total examination such cysts appear to be entirely uncovered on all or part of their exposed surfaces (figs. 7, *a*, and 8, *b*). When sectioned, however, this proves to be illusory and none of the glochidia actually shows bare areas. The cyst wall is merely very thin (fig. 6). Now, this is exactly what happens in normal encystment (Arey, '32 *a*), except that in immunity the thinning is more likely to be carried to an extreme.

The query immediately presents itself as to what becomes of the cells that formerly composed the thick cyst. It is natural to assume that the reduction depends on a direct cellular loss by sloughing, yet actual microscopic observation does not confirm this view. It must be remembered that gill cysts are made up of cellular connective tissue overlaid with epithelium (fig. 1). As the size reduction goes on there is no evidence of epithelial necrosis, loosening, or desquamation. Hence, so long as the continuity of the epithelium is maintained there can be no sloughing of the underlying stroma cells (fig. 6). What, then, does occur? As the cyst becomes smaller its contour also grows smoother. Both of these occurrences appear of necessity to depend on the movement of cells from the cyst wall backward into the body of the filament. Such a process is in a sense not surprising, for it is merely the reverse of the outward migration at the time of encystment. But now the response is harder to comprehend, for there lacks the visible stimulus that originally called the cellular elements forth.

Finally, comes the problem of actual shedding, whereby intact glochidia, as well as the valves and cellular debris of destroyed ones, separate from the filament. Frequently the cyst reshapes itself until it is distinctly stalked (fig. 5), and, together with its contents, is lost as a whole. Yet this cannot be the commonest manner of removal. The majority of glochidia become thinly covered while retaining the original

broad attachment. They are then cast off, but as to what process directly precedes the loosening I am not able to state, for illustrative stages have not been observed. In some instances there is evidence of undercutting, yet this cannot be the regular method, since the broad attachment still continues as a rule in filaments from which glochidia are already being lost. Microscopically there were no noticeable changes about the bases of such cysts as have been sectioned or examined totally (fig. 6). Against these statements must be placed the fact that glochidia undeniably are got rid of in some manner. The details await further inquiry which must combine observations on living filaments with subsequent microscopic examination. It is my belief that the intact or disintegrated glochidium is cast off within the highly thinned cyst as a unit. Empty cysts have not been observed, whereas deeply scalloped filaments are common (figs. 7 and 9).

Two somewhat incidental statements are on record which are of interest inasmuch as they refer to living conditions. Reuling ('19), also utilizing black bass made immune to *Lampsilis luteola*, records: "On examination after 24 hours the gills of the fish which had previously been infected twice showed marked necroses and sloughing of the epithelial [sic] cyst around each glochidium." Howard ('14), working with the *Quadrula* group, refers to the reaction when glochidia find attachment to a non-host and says: "If not [retained], the cyst is shed by a process of desquamation of the external epithelium. I have observed this in gill infections only. A stream of water of not great force will remove the outer layers of epithelium of an infected gill about to shed the glochidia. Such a catarrhal reaction. . . ."

For a short time after glochidia are shed the filaments appear notched, each scallop indicating clearly the former location of a glochidium (figs. 7 and 9). When glochidia are closely spaced a solid stretch of filament tissue is lost with them and as a result the filament for a time is greatly narrowed locally (fig. 7, at tip). In a surprisingly few hours the filament is restored to its normal size and shape.

SUMMARY

Glochidial cysts on the gills of immune fishes form in the same manner as normal ones, but they tend to grow larger and become more irregular. The increased thickness is due to additional cellular connective tissue in the wall.

The gill tissue indicates the existing biological incompatibility only by the presence of eosinophiles, extruded chromatin spherules, and eosinophilic plastids.

In natural, or racial immunity many glochidia are promptly destroyed by cytolysis, accompanied by an invasion of host cells. These disintegrating glochidia may occur in close proximity to unaffected glochidia and apparently are merely less resistant individuals that succumb to a critically adjusted reaction.

In both natural and acquired immunity the normal retention of glochidia and the accompanying metamorphosis are replaced by premature shedding. After the first day, the cyst thins by the removal of stroma cells back into the filament until the wall is reduced to a thin envelope. Both intact and destroyed glochidia, and apparently their cyst coverings, are sloughed at about the second day. Repair of the resulting notched filament is prompt.

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PLATE 1

EXPLANATION OF FIGURES

1 Section through a typical immune cyst of *Lampsilis luteola*, nine hours after attachment. The glochidium is surrounded by a zone of eosinophiles. Photo. $\times 147$.

2 Section through neighboring normal (at right) and degenerating (at left) glochidia of *Lampsilis anodontoides*, fifteen hours after attachment. Among the remains of the opened, degenerating larva, the hinge (*h*), cuticula, and parts of the mantle are distinguishable. Photo. $\times 167$.

3 Two opened and partly degenerated glochidia of *Lampsilis anodontoides* at twenty-one hours. A piece of the disintegrating adductor muscle is seen on each lateral side of the right-hand larva. Photo. $\times 167$.

4 Two late stages in the degeneration of *Lampsilis anodontoides* glochidia after forty-two hours of encystment. In the widely opened larva on the right, the hinge (*h*) and mantle cuticula are alone recognizable; in the cyst at the left the hinge appears in another section. The two cysts communicate, but are partly separated by an incomplete partition. Photo. $\times 167$.

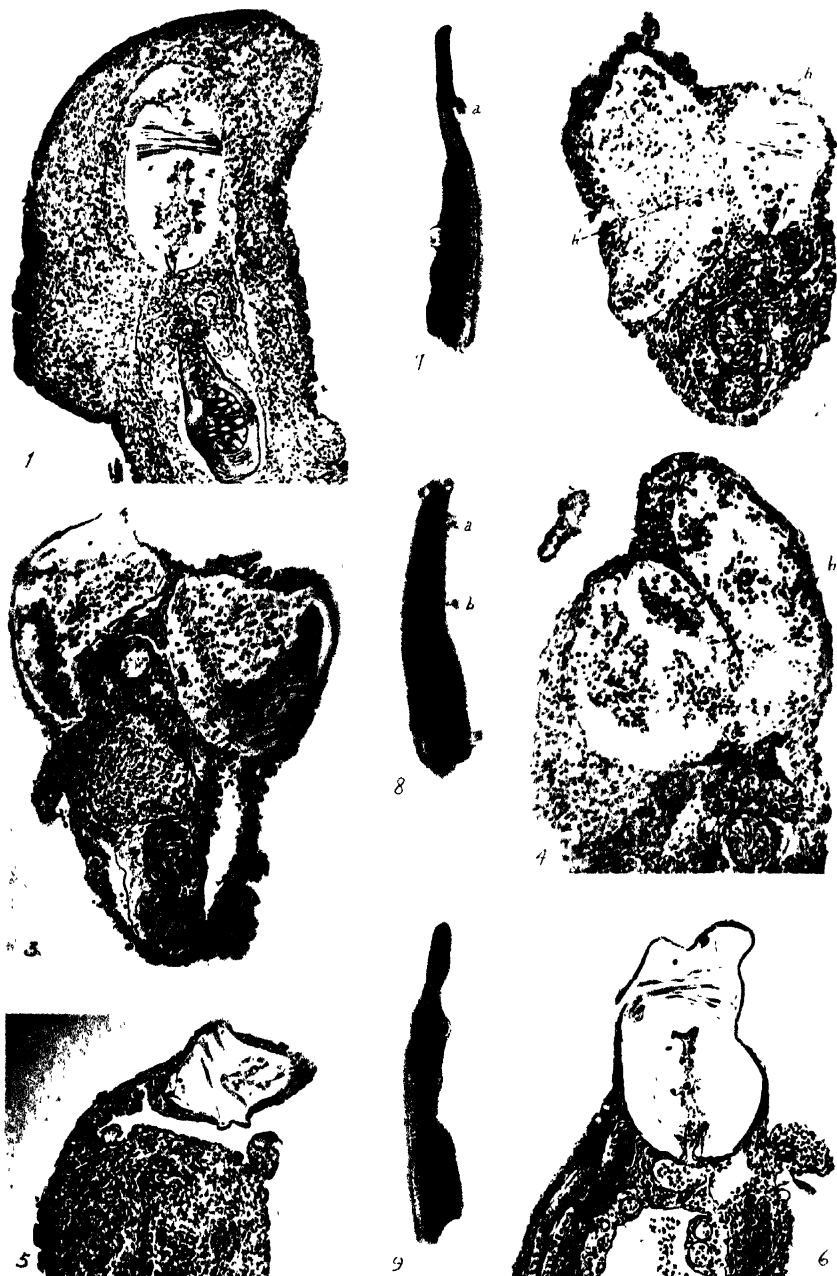
5 A forty-hour pedunculated cyst of *Lampsilis anodontoides*. The section passes obliquely through the glochidium. Photo. $\times 167$.

6 A greatly thinned cyst of *Lampsilis luteola*, fifty hours old, nearly ready to slough. Photo. $\times 167$.

7 Gill filament with fifty-hour cysts of *Lampsilis luteola*. The cyst designated *a* is unevenly reduced in thickness. Extensive sloughing has carried away much tissue from the distal half of the filament. Photo. $\times 17$.

8 Gill filament with sixty-seven-hour *Lampsilis luteola* cysts. At *a* the cyst has thinned unequally; at *b*, so evenly and thoroughly that the glochidium appears to be naked. Photo. $\times 17$.

9 Gill filament with a sixty-seven-hour *Lampsilis luteola* cyst and several notches indicative of previously sloughed glochidia. Photo. $\times 17$.



THE STRUCTURE OF OPALINIDS, AS REVEALED BY THE TECHNIQUE OF MICRO-INCINERATION¹

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ONE FIGURE

AUTHORS' ABSTRACT

Opalinids from the rectum of *Rana pipiens* were isolated in large numbers and fixed in a mixture of nine parts of absolute alcohol and one part of neutral formalin. These were embedded in paraffin and incinerated according to the technique of Polcard. Examination of the preparations by dark-field illumination showed that the micro-incinerated protozoa retained their distinctive morphological and cytological characters, for the mineral ash had the same topographical distribution as the cell inclusions in the control stained preparations.

The coarse vegetative granules of the cytoplasm were preserved as distinct masses of ash which corresponded exactly to these bodies in the control preparations. The structure of the myonemata and the cilia was perfectly preserved. The chromatin material of the nucleus, more or less abundant, left little or no ash, which is quite in contrast to the findings of Scott with regard to amphibian and mammalian nuclei.

Investigators who had interested themselves in the functional significance of the Golgi bodies and associated cytoplasmic structures first discovered in the cells of higher animals, later turned from the vertebrate cell to a study of the protozoa with the hope of discovering the ancestral homologue of these varied and complex structures. Several authors have confused the issue by describing certain specialized structures found only in the protozoa as representing true Golgi elements. Of late, Nasonov ('24), pursuing a similar line of inquiry, has attempted to prove a relationship between the contractile vacuole of certain protozoa and the Golgi apparatus of the higher organisms, while Duboseq and Grassé ('25) have added to the controversy by contending that the parabasal bodies of flagellates represent Golgi elements. In addition, King and Gatenby ('26) have further described a relationship between the cilia and certain pyri-

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form osmiophile structures in the endoplasm of opalinids, which they likewise presume to be Golgi material.

In order to help elucidate certain of these findings in the opalinids, discovered by somewhat capricious techniques, it was deemed advisable to employ the micro-incineration technique of Policard ('29), by which the inorganic structure of the whole organism can readily be determined.

MATERIAL AND TECHNIQUE

It was necessary to use a fixative which neither added to nor detracted from the mineral elements of the cells in question. Absolute alcohol met these requirements, but gave poor fixation even under the most favorable conditions. It was found that a mixture of nine parts of absolute alcohol and one part of neutral formalin (Will Corporation) gave reasonably good fixation and yet caused no detectable difference in the ash deposits.

Opalinids infecting the rectum of *Rana pipiens* were isolated in large numbers and bathed in physiological saline prior to fixation in the above mixture. After fixation for twenty-four hours, the organisms were passed through several changes of absolute alcohol in order to complete the dehydration and remove the formalin. The material was then cleared in xylol, embedded in paraffin, and sectioned at 4μ . The sections were smoothed out upon an ordinary glass slide with the aid of a drop of liquid petroleum, necessary care being taken to avoid contact with water. The preparations were then incinerated in a special electric quartz oven at a temperature of 625°C . for periods ranging from twenty-five to forty-five minutes. The incinerated sections were mounted in the dry manner and examined under the microscope with dark-field illumination secured by employing a Zeiss cardioid condenser, 3-mm. iris objective, and binocular attachment. The micro-incinerated organisms were compared with control opalinids, which had either been fixed in the above fixative or else treated with the chrome-osmic technique, prior to staining with Heidenhain's iron haematoxylin and occasional counterstaining with eosin.

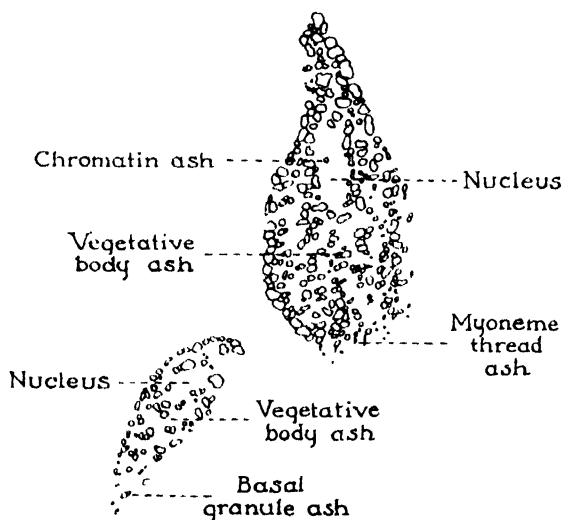


Fig.1 Upper: Dark field photomicrograph of incinerated sections of opalinids; Zeiss apochromatic 20 N.A. 0.65, 10 $\times$ ocular, and cardioid condenser. Lower: Superimposed drawing of the above photograph, giving the description of the various structures.

The ash of incineration retained the same form as in the fixed opalinid (fig. 1), thus giving a mineral skeleton of the protozoan cell. All inorganic salts common to the organism and which were not volatilized by the heat of incineration remained in their original position. Iron, when present, assumed the form of iron oxide and was easily recognized by its color. Calcium was in all probability present as an oxide, but could not be differentiated optically from other salts. Silicates were recognizable because of their birefringence when viewed with polarized light.

CYTOLOGY OF CONTROL ORGANISMS AND RÉSUMÉ OF PREVIOUS WORK

Before attempting to describe the appearance of the incinerated opalinids, it will be as well to give an account of the general cytology of the control organisms and also a brief résumé of the controversial literature upon the subject.

Examination of the control infusorians after a chromosmic fixation reveals deeply staining rod-like mitochondria, aggregated in the endoplasm, which maintain a precise longitudinal orientation within the peripheral areas of the cell. Besides the mitochondria, many coarse, faintly staining granules, which may be either pyriform, spherical, or quite irregular in shape, are also seen scattered at random within the general cytoplasm. Many of these characteristic cell granules are detected either in the isolated condition or else lying in close connection with the surface of a mitochondrion, as has been previously described by Richardson and Horning ('31). These authors have demonstrated that these granules arise in connection with the cell mitochondria and when fully formed become dissociated and migrate to positions within the protoplasm as free structures until they finally become absorbed ('25).

Bearing in mind the method of feeding of this astomatous ciliate and the conception of the enzymatic nature of mitochondrial activity ('27), it has been suggested that these associated granules are synthesized under the influence of the mitochondria. Experiments on the variation of the

metabolism of *Opalina* (Richardson and Horning, '31) as well as on the behavior of these granules during the life cycle of the organism also suggest their vegetative nature (Horning, '25), and for the sake of convenience we shall refer to these structures as 'vegetative' granules.

Further observations reveal that the vegetative grains after fixation in the alcohol-formalin fixative and treatment with the Heidenhain stain show up clearly and present a similar morphology to those seen in sections treated with the chrome-osmic technique. The accompanying mitochondria, however, as one would expect, do not show a selective absorption for the stain, as they have apparently been dissolved out by the absolute alcohol contained in the fixative employed.

King and Gatenby ('26), employing a Champy-osmium technique, have already referred to certain pyriform structures in the endoplasm of *Opalina rarum* which they designate as osmiophile bodies and presume to be of the nature of Golgi bodies. They further contend that these pyriform Golgi bodies in the majority of cases lie in direct contact with the cilia, thereby suggesting an homology between the parabasal structures of flagellates, which Duboseq and Grassé ('25) hold consist of Golgi substance, and the coarse pyriform osmiophile bodies of *Opalina*.

Sokolska ('27) has similarly described these 'vegetative bodies' in preparations of *Opalina* following the chrome-osmic method, and has likewise contended that they represent Golgi bodies. This author, however, differs from King and Gatenby in her interpretations. She describes faintly staining granules which lie in close association with what we interpret as the mitochondria orientated longitudinally, but suggests that they are merely osmiophile bodies encircling a 'bound secretion' and are therefore of Golgi nature. Sokolska, unlike King and Gatenby, failed to indicate any relationship between the coarse polymorphic granules and the cilia. In a recent publication Richardson and Horning ('31), using Cajal and da Fano techniques, showed the Golgi substance to be independent from both the vegetative grains and the mitochondria. Furthermore, no connection could be detected

between the cilia and the vegetative structures, such as King and Gatenby describe ('26). In our control preparations each single cilium was found to terminate in a small basal granule in such a manner as has been previously described by Minchin ('22) as characteristic of the ciliate Infusoria.

CYTOLOGY OF THE MICRO-INCINERATED ORGANISMS

Bearing in mind this controversy concerning the cytology of the opalinids, it is especially interesting to reinvestigate this problem from an entirely new aspect.

The micro-incinerated protozoa, when observed by dark-field illumination, are seen to retain their distinctive morphological and cytological characters, for the mineral ash deposit bears the same topographical distribution as the cell inclusions in the control preparations. One great advantage of this technique, apart from the fact that artefacts are not likely to be produced, is that it opens up a new and interesting field by which the inorganic analysis of the various constituents of the cell can readily be developed. Sections of incinerated opalinids show that the coarse vegetative granules are characterized by the appearance of well-outlined accumulations of fine mineral ash, which correspond exactly, not only in their distribution, but also in their contours, with those of the non-incinerated control preparations, as an examination of the photomicrograph will demonstrate.

The rod-like structures, which we have previously designated as mitochondria and which are frequently seen lying in association with these polymorphic granules in chrome-osmic preparations, are not detectable in the micro-incinerated material. The mitochondrial nature of these inclusions is again suggested, inasmuch as they have apparently been dissolved out in the absolute-alcohol fixative prior to incineration. Likewise, it may be assumed that if these vegetative granules were of Golgi substance, they would have suffered the same fate as the mitochondrial material. Close examinations, under the high powers, of well-incinerated surface sections of the opalinids actually reveal the inorganic structure of the myonemata in the form of a very delicate, fine mineral

ash deposit orientated in delicate lines running parallel to the axis of the cell (see photograph). The order as well as the proximity of the myoneme threads to one another in the micro-incinerated preparations compares favorably with those of the controls.

Other minute bodies also readily revealed by this technique, and seen to advantage in sections cut through the middle of the organisms, are the basal granules which are located within the peripheral regions. The remaining inorganic deposit of these small structures is clearly detected when using an oil-immersion objective; however, these clumps of mineral ash lie so near to each other and are so minute that when observed with dark ground light under the lower powers of the microscope, they give the appearance of one continuous outline. The persistence of these bodies after incineration is of interest, as King and Gatenby ('26) pictured the cilia terminating in the vegetative bodies of the opalinids. Evidence taken from both the micro-incinerated and control material shows them clearly to be separate independent structures.

The nuclei of the opalinids showed a peculiarity which apparently has thus far been unnoticed. The chromatin material, although more or less abundant, showed almost no ash residue. This was quite in contrast with the finding of Scott ('30 a, b, and c) in the nuclei of acinar and duct cells of the submaxillary gland of the guinea-pig and in the epidermal cells of the tadpole. In these instances there was quite a distinct ash deposit in the nucleus which corresponded remarkably well with the location of the chromatin material in stained control sections. In general, it may be stated that one of the most striking features in an incinerated preparation of mammalian tissue is the clearness and amount of the nuclear ash deposit. The lack of ash in the nucleus of the opalinid is also of interest, especially in connection with the results obtained by Lucas ('30) in applying the Feulgen reaction to these organisms. The thymonucleic-acid reaction was quite as intense in these infusorians as in mammalian or amphibian tissues.

Lying adjacent to the basal granules, bordering the periphery of the cell, incinerated ash representing the inorganic remains of the cilia was frequently observed in sections cut through the middle of the ciliate. The inorganic structure of the cilia consists of an extremely fine mineral salt deposit beautifully orientated in a radial manner from the body of the cell. The perfect inorganic remains of these very delicate structures demonstrates the advantages of the micro-incineration method, not only as a histochemical, but also as a cytological technique.

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THE EFFECTS OF X-RAYS ON REGENERATION IN TUBIFEX TUBIFEX¹

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FIVE PLATES (TWENTY-SIX FIGURES)

AUTHOR'S ABSTRACT

Posterior regeneration in *Tubifex* is inhibited by suitable radiation with x-rays. Only a small knob is formed at the cut surface by rearrangement of the terminal region of the old body tissues. Location of the cut within the segment and repeated removals of segments within the posterior three-fourths of the body do not change this result. The worms are as though 'castrated' against regeneration. Normal worms regenerate readily under similar conditions and replace lost segments within thirty-five days. Mesodermal tissues in normal regenerating *Tubifex* are formed from neoblasts, which arise from peritoneal cells upon the posterior faces of septa near the cut, migrate to this wound surface, and differentiate into new structures. After radiation no neoblasts arise from peritoneal cells and there is no mesodermal regeneration. No changes, other than failure to form neoblasts, can be observed in the peritoneal cells. Migrating neoblasts are destroyed within a few hours by similar radiation.

Epithelial tissues are also affected by x-rays, as shown by absence of mitoses and failure of regeneration in ectodermal and endodermal epithelia. During normal regeneration cells which form these epithelia and certain muscle fibers arise by proliferation from the epidermis and intestinal lining in the regenerating region, as shown by numerous mitoses in these layers. Failure of regeneration in radiated worms is thus related to lack of cells which are present in normal regeneration.

INTRODUCTION

During regeneration in some annelids new mesodermal tissues are formed from undifferentiated cells which Randolph ('92) called 'neoblasts.' The importance of these cells during regeneration has been confirmed by later workers, and Kreckler ('23) gives an account of the cycle of neoblast activity in *Tubifex* and *Limnodrilus*. Similar reserve cells have been described in other forms, and their importance as a source of new material in regeneration is the basis for the hypothesis that plants and animals possessing such cells with embryonic potentialities are the ones capable of maximum powers of regeneration during their adult stages.

¹ This investigation is one of the studies upon effects of radiation upon regeneration in progress in the department of zoölogy at the University of Missouri, assisted by grants to W. C. Curtis from the Committee on Effects of Radiation of the National Research Council.

The value of x-rays and radium emanations as a technique of experimentation with living organisms was recognized soon after the discovery of the differential susceptibility of tissues to these radiations. Packard ('31) has recently reviewed the subject. Schaper ('04) showed that Triton larvae fail to regenerate after exposure to radium emanations. Recently, Curtis and Hickman ('26) found that *Planaria agilis* failed to regenerate following the destruction of the so-called formative cells by x-rays, while Zawarzin and Strelin ('28) obtained similar results with *Pelmatohydra*, and Weigand ('30) records a failure of regeneration in *Planaria* and *Clavelina* after treatment with radium emanations. The similarity in function between the neoblasts of annelids and the formative cells of planarians suggested that the formation of new mesodermal structures in annelid regeneration would be inhibited if the neoblasts could be destroyed or prevented from reaching the regenerating end by radiation. *Tubifex tubifex* was chosen, as previous workers had reported the presence of neoblasts during regeneration in this species.

MATERIAL AND METHODS

The *Tubifex* used in these experiments were found in great numbers along the edges of old ponds. The worms were kept in running tap water during the period of regeneration. For operation and later observation they were anesthetized in 0.22 per cent chloretone solution. The segments were cut off with a small scalpel and the regeneration studied under a binocular microscope. The amount of new tissue is clearly seen in the living worms and can be stated exactly in terms of segments, since the new segments are recognizable from early stages by greater transparency, lesser diameter and length, and by absence of the pigmented chloragogue cells which cover the old intestine.

During radiation the worms were always covered by the same depth of the chloretone solution. The anesthetic causes them to lie extended on the bottom of the dish, so that maximum uniformity of exposure to the rays may be expected.

The Petri dish containing the worms was placed 8 inches below the center of the target of the x-ray tube and covered with a thin sheet of cardboard to screen the light and heat. The exposures were continuous for twenty-five to thirty minutes as follows:

Tube current,	4 m.a.
Voltage,	140 K.V.
P.K.V.,	205-210
Distance,	8 inches
r unit intensity per minute,	237.5

The worms were fixed in Gilson's mercurio-nitric fluid and in Heidenhain's trichloroacetic mixture. Whole mounts of the regenerating region were stained with alum cochineal. Sections were cut 5μ and stained with Delafield's haematoxylin and iron haematoxylin with orange G.

OBSERVATIONS AND EXPERIMENTS

Before attempting radiation experiments with Tubifex it was necessary to verify the accounts of older workers. In addition to showing that Tubifex was suitable for this investigation, these preliminary experiments were also of value in checking the influence of other factors upon regeneration, such as the varying conditions to which the worms might be exposed in laboratory. In the experiments summarized in table 1 the effects of temperature changes, exposure to chloretone solution, and of anterior regeneration occurring simultaneously with posterior regeneration were compared.

As shown in groups *a* and *b*, variation in temperature from 18.5°C. to 22°C. had little effect upon the rate or amount of regeneration. It was necessary to anesthetize all the worms in 0.2 per cent chloretone solution for three to five minutes every third day to count the new segments. In group *c* the regeneration was not markedly affected, if at all, by ten minutes' daily immersion in such a chloretone solution, and it is unlikely that the results in further experiments would be altered by the shorter exposure at three-day intervals. The simultaneous formation of new anterior segments shown

in *d* had little if any effect upon posterior regeneration, since the average number of new posterior segments formed in *d* is nearly the same as in *a* and *b* in which the anterior segments were not removed. These data support the observa-

TABLE 1

Comparison of posterior regeneration in normal worms under different conditions. The worms were kept in crystallizing dishes of 9-inch diameter containing tap water. All were anesthetized for a few minutes every third day to count the new segments. Worms that failed to regenerate together with those that crawled out of the dishes and died were not counted in computing the average number of new segments. Experiments discontinued at twenty-three days on account of protozoan infection

CONDITIONS OF EXPERIMENT	WORMS AT BEGINNING	NEW SEGMENTS AT TWENTY-ONE DAYS	AVERAGE NUMBER NEW SEGMENTS IN REGENERATING WORMS	DIED OR LOST DURING EXPERIMENT	WORMS NOT REGENERATING AT TWENTY-ONE DAYS	WORMS REGENERATING AT TWENTY-ONE DAYS
<i>a.</i> Worms in standing water, 22°C. average temperature, water changed daily, posterior ends removed	30	540	22.5	2	4	24
<i>b.</i> Worms in running water, 18.5°C. average temperature, posterior ends removed	30	542	20.8	2	2	26
<i>c.</i> Worms in running water as in <i>b</i> , immersed daily for 10 minutes in 0.2 per cent chloretone solution, posterior ends removed	30	551	25	3	5	22
<i>d.</i> Worms in running water as in <i>b</i> , anterior and posterior ends removed	30	495	20.6	3	3	24

tions by Hescheler ('98) in Lumbricidae and Muller ('08) in *Tubifex rivulorum*, that the anterior and posterior ends of annelids regenerate independently of one another.

Regeneration after exposure to x-rays

A series of exposures to increasing intensities of x-rays was undertaken to discover if there was an effect upon regeneration and the amount of radiation necessary to produce

this effect. After a four-day latent period, fifteen to twenty posterior segments were removed. The total number of segments replaced by each group is given in table 2 for three-day intervals up to thirty-three days. With increasing exposure there is a progressive decrease in the number of new segments formed until only an occasional worm will regenerate after twenty-five minutes' radiation. The thirty-minute exposure inhibits regeneration in all individuals. In the hundreds of worms used in later experiments no regeneration was ever observed after a thirty-minute exposure, whereas in the controls of this experiment thirty-seven worms formed a total of 1189 new segments in thirty-three days. The radiated worms formed only an 'anal knob' consisting of a terminal portion of the body approximately half the length of a normal segment (figs. 3, 4). In the transparent living worms the intestine can be seen extending through this region to the anal opening. The anal knob is formed within five or six days after the operation, apparently by rearrangement of the old body tissues adjacent to the cut surface, since the tip never has the enlarged opaque appearance characteristic of normal regenerating worms at this stage (figs. 1, 2). The normal worms form during their regeneration a series of new segments and septa at the posterior end (figs. 3, 4). The first segments formed adjacent to the old tissue increase in length and diameter with age and a series of progressively smaller new segments extends back to the proliferating region near the tip. As the process approaches completion, segment formation becomes less active and finally ceases while the new segments grow to normal size. The new tissue can be recognized in most instances for a considerable period after the segments have grown to adult size by the absence of the brown chloragogue cells from the regenerated intestine.

Numerous repetitions of the foregoing experiment have given similar results. Table 3 is a summary of one such experiment in which four of the radiated worms lived without regeneration 147 days after removal of the posterior ends.

TABLE 2

Posterior regeneration after exposure to x-rays for varying periods. The 9-, 12-, 15-, and 18-minute results are from one experiment and the 21-, 25-, 30-minute, and control results are from another. The controls in both experiments gave practically the same results. A four-day latent period was used

CONDITIONS OF EXPERIMENT	WORKS AT BEGINNING	TOTAL NUMBER NEW SEGMENTS FORMED IN DAYS INDICATED											AVERAGE NUMBER NEW SEGMENTS	DIED DURING EXPERIMENT	WORKS AT END	WORKS REGENERATING
		3	6	9	12	15	18	21	24	27	30	33				
a. X-rayed 9 minutes: 15 to 20 posterior segments removed	40	0	0	9	11	17	18	18	21	25	1	1	.74	9	31	7
b. X-rayed 12 minutes: 15 to 20 posterior segments removed	50	0	0	0	6	18	18	19	21	23	24	31	.77	10	40	6
c. X-rayed 15 minutes: 15 to 20 posterior segments removed	40	0	0	0	2	5	5	6	6	7	7	7	.24	11	29	3
d. X-rayed 18 minutes: 15 to 20 posterior segments removed	35	0	0	0	0	2	7	10	14	17	17	18	.66	8	27	5
e. X-rayed 21 minutes: 15 to 20 posterior segments removed	60	0	0	0	0	0	0	0	0	2	2	2	.04	12	48	1
f. X-rayed 25 minutes: 15 to 20 posterior segments removed	50	0	0	0	0	0	0	0	0	2	4	4	.09	9	41	1
g. X-rayed 30 minutes: 15 to 20 posterior segments removed	110	0	0	0	0	0	0	0	0	0	0	0	0	23	87	0
h. Controls: 20 to 30 posterior segments removed	45	69	201	313	551	729	830	903	976	1057	1138	1189	32.1	8	37	37

¹ Protozoan infection destroyed the culture.

TABLE 3
Comparison of regeneration in x-rayed and control worms

CONDITIONS OF EXPERIMENTS	TOTAL NUMBER NEW SEGMENTS FORMED IN DAYS INDICATED											WORMS AT BEGINNING	WORMS NOT REGENER- ATING AT THIRTY DAYS	WORMS REGENERATING AT THIRTY DAYS
	3	6	9	12	15	18	21	24	27	30	62	94	147	
X-rayed 30 minutes: Posterior ends re- moved five days later	52	0	0	0	0	0	0	0	0	0 ¹ (47)	0 ¹ (25)	0 ¹ (23)	0 ¹ (4)	47
Controls: Posterior ends removed as above	72	0	270	757	1029	1560	1741	1817	1923	1995	2018	2	32	1
														65

¹ There were 47, 25, 23, and 4 specimens of the radiated worms alive as indicated, none of which regenerated.

² Junction of old and new segments indistinguishable after thirty days.

The radiated worms decreased in size during this period and at the end were approximately half as large as at the beginning. The controls formed an average of thirty-two new segments in thirty days (fig. 5), after which it was impossible to distinguish clearly the boundary between old and new tissues. It is evident from these experiments that a sufficient exposure to x-rays will inhibit posterior regeneration in *Tubifex* in agreement with the observations of Curtis and Hickman ('26) in *Planaria* and of Zawarzin and Strelin ('28) in *Pelmatohydra oligactis*.

Regeneration after cuts through different regions of a segment

It is possible that regeneration in *Tubifex* may be influenced by the region of a segment through which the cut passes, on account of differences in the rate of wound closure and initiation of regeneration. Hescheler ('96) found in the Lumbricidae that portions of segments are constricted off at the adjacent septum before regeneration begins. In order to discover what the behavior of *Tubifex* would be under these conditions, worms were anesthetized and cut with a small scalpel at different levels in the segment. The total number of segments regenerated by groups of worms after such cutting is shown in table 4. In no case was there any regeneration in the x-rayed worms. They formed the usual anal knob from the terminal portion of the segment without loss of any tissue. The controls likewise retained the parts of segments, and regenerated at practically the same rate regardless of the location of the cut. Since the regeneration does not seem to be influenced by the location of the cut within the segment, this factor may be disregarded.

Regeneration from different regions of the body

There have been numerous experiments by earlier investigators upon the regenerative power of annelids when severed at different regions of the body. In general, the rate and amount of regeneration depend upon the location of the cut

in relation to the anterior end. While the more anterior portions of the body will form a greater number of posterior segments if allowed sufficient time, a certain number of segments must be present for regeneration to occur at all. Thus Morgan ('97) and Michel ('99) in *Allolobophora* found irregular, reduced regeneration when the anterior piece consisted of thirteen to thirty segments and no regeneration if less than thirteen segments were present. Haase ('98) and Muller ('08) found that anterior ends of *Tubifex* must contain at least ten segments before regeneration would occur, but such pieces regenerate very imperfectly.

The rate of regeneration from different levels of the body is shown in table 5. The radiated worms failed to regenerate and formed the characteristic anal knob in all cases, although formation of this structure was much slower when three-fourths of the body was removed. The controls regenerate at practically the same rate when one-fourth or one-half the body is severed, and the only difference is in the length of time necessary to replace all the lost segments. The rate of regeneration in the controls, in which only the anterior quarter consisting of thirty to forty segments remains, is not more than one-third that of the other groups and a correspondingly longer period of time is necessary to complete the process. None of these pieces replace all the lost segments. Removal of additional segments retards still further the onset and rate of regeneration, and pieces consisting of fifteen to eighteen anterior segments seldom regenerate. These results agree with the conclusions of previous workers as to the rate and amount of posterior regeneration from different regions of the body and demonstrate that the failure of regeneration in radiated worms is not influenced by the level of the body at which they are severed.

Regeneration after successive removals of posterior segments

Successive posterior regenerations in normal worms have been obtained by a number of investigators. Muller ('08), in *Tubifex*, was able to obtain forty-two posterior regenera-

TABLE 4
Comparison of x-rayed and control worms after removal of posterior ends by cuts through different regions of a segment

CONDITIONS OF EXPERIMENT	WORKS AT BEGIN- NING	TOTAL NUMBER OF NEW SEGMENTS FORMED IN DAYS INDICATED												WORKS AT END	AVERAGE NUMBER NEW SEGMENTS	NUMERICAL RANGE OF NEW SEGMENTS
		3	6	9	12	15	18	21	24	27	30	33				
X-rayed 25 minutes: Posterior end severed at a septum	15	0	0	0	0	0	0	0	0	0	0	0	11	0	0	
Controls: Posterior end sev- ered at a septum	8	0	11	38	84	103	129	130	130	132	133	133	7	19	6-36	
X-rayed 25 minutes: Posterior end severed at middle of segment	15	0	0	0	0	0	0	0	0	0	0	0	15	0	0	
Controls: Posterior end sev- ered at middle of segment	8	0	9	26	43	55	70	77	91	108	121	127	7	18	5-27	
X-rayed 25 minutes: Posterior end severed at posterior one-fourth of segment	15	0	0	0	0	0	0	0	0	0	0	0	12	0	0	
Controls: Posterior end sev- ered at posterior one-fourth of segment	8	0	14	21	71	79	83	91	111	123	130	137	8	17	9-25	

TABLE 5

Comparison of regeneration in x-rayed and control worms after removal of different portions of the body

CONDITIONS OF THE EXPERIMENTS	WORMS AT BEGINNING	TOTAL NUMBER OF NEW SEGMENTS (DAYS)										AVERAGE NUMBER NEW SEGMENTS TWENTY- SEVEN DAYS	WORMS ALIVE AT END	DIED DURING EXPERI- MENT
		3	6	9	12	15	18	21 ¹	24 ¹	27				
X-rayed 25 minutes: Posterior one-fourth of body removed Controls: Posterior one-fourth of body removed	20	0	0	0	0	0	0	0	0	0	0	19	1	
X-rayed 25 minutes: Posterior one-half of body removed Controls: Posterior one-half of body re- moved	20	0	86	198	285	343	362	...	...	418	22	19	1	
X-rayed 25 minutes: Posterior three- fourths of body re- moved Controls: Posterior three-fourths of body removed	20	0	0	0	0	0	0	...	...	0	0	19	1	
X-rayed 25 minutes: Posterior three- fourths of body re- moved Controls: Posterior three-fourths of body removed	20	0	61	145	271	323	372	...	...	417	24.5	17	3	
X-rayed 25 minutes: Posterior three- fourths of body re- moved Controls: Posterior three-fourths of body removed	20	0	0	0	0	0	0	...	...	0	0	16	4	
X-rayed 25 minutes: Posterior three- fourths of body re- moved Controls: Posterior three-fourths of body removed	20	0	33	81	117	140	161	...	...	234	13	18	2	

¹ New segments not counted for these periods. Experiment discontinued due to protozoan infection at twenty-nine days.

tions from a single worm over a period of nine months, and other workers have reported similar results in other forms. In order to determine whether the radiated worms would recover their regenerative powers when subjected to the

TABLE 6

Regeneration after successive removals of posterior segments. The indicated number of old segments, plus the regenerated segments, were removed in the controls at successive operations

CONDITIONS OF THE EXPERIMENTS	WORMS AT BEGIN- NING	TOTAL NUMBER OF NEW SEGMENTS IN PERIOD INDICATED				WORMS ALIVE AT END OF EACH PERIOD	AVERAGE NUMBER OF NEW SEG- MENTS	DIED DURING EACH PERIOD	NUMERICAL RANGE OF NEW SEG- MENTS
		7	14	32	49				
a. X-rayed 25 minutes: 15 to 20 posterior segments removed	20	0				19	0	1	0
b. Controls: 15 to 20 posterior segments removed	20	73				20	3.5	0	2-6
a. X-rayed: 10 to 15 additional posterior segments removed	19		0			18	0	1	0
b. Controls: 10 to 15 additional posterior segments removed	20		67			19	3.5	1	1-6
a. X-rayed: 10 to 15 additional posterior segments removed	18			0		11	0	7	0
b. Controls: 10 to 15 additional posterior segments removed	19			241		11	15	3	9-25
a. X-rayed: 6 to 10 ad- ditional posterior seg- ments removed	11				0	7	0	4	0
b. Controls: 6 to 10 ad- ditional posterior seg- ments removed	16				41	14	2.6	2	1-6

stimulus of repeated removals of posterior segments, the experiment summarized in table 6 was undertaken. The controls were capable of regeneration after each amputation. The average number of new segments formed in the last seven-day period after the fourth successive removal of pos-

terior segments was less than that of the first period, but this would be expected in view of the reduced number of anterior segments remaining and the amount of new tissue already formed by the worms in the preceding periods with no food available. The higher average regeneration for the third (eighteen-day) period is probably due to the reduced proportion of that period which the preliminary stages (lasting two to four days) occupy. After these preliminary stages there follows a rapid formation of new segments which lasts fifteen to eighteen days; the higher average for the eighteen-day period can be explained by the greater portion of that period which falls within these limits. The numerical range of the new segments in each group of controls is practically the same for corresponding periods of time. The x-rayed worms formed only the anal knob after each amputation and the additional stimulus of successive removals caused no regeneration.

On account of the complete absence of regeneration in *Tubifex* receiving a sufficient exposure to x-rays under the various experimental conditions presented in tables 2 to 6, we may conclude that such radiation brings about a permanent loss of regenerative power in this species. The *Tubifex* that have been x-rayed are as though 'castrated' against regeneration.

INTERNAL CHANGES

Normal worms

Worms were sectioned longitudinally at twenty-four-hour intervals to determine the stages in replacement of the lost segments and the sources of new material. The structural changes of the first twenty-four hours are not regenerative, since they are mainly related to closure of the wound. Six hours after the operation, in approximately half the specimens the body wall appears as though constricted by muscle contraction at the cut surface, so that the epidermis begins to meet and fuse with the edges of the gut wall without closure of the digestive tract. There are numerous injured muscle cells mingled with granular cells in the body cavity adjacent

to the wound region. In the remainder of the specimens the intestine retracts for a short distance and closes to form a blind tube. The space posterior to the closed end of the intestine is filled by a loose mass of injured muscle cells from the severed muscles of the body wall and granular cells from the body cavity. This cicatrix, together with the constricted epidermis, serves to close the body cavity, while individual epidermal cells are pushing over the outer surface of the wound to form a continuous layer. Twelve hours after the operation, fusion of the epidermis and gut wall is complete in the worms in which the intestinal lumen is not closed, and the cut ends of the body-wall muscles lie against the outer surface of the intestine adjacent to the anal opening. The cicatrix is still in position between the edges of the severed body-wall muscles in worms in which the closed intestine is retracted and the epidermis forms a continuous layer of flattened cells over the outer surface of the wound, apparently by migration of the old cells, since no mitotic figures are present in this layer. These observations agree with the description of Wagner ('00, '06) in *Lumbriculus variegatus* with regard to fusion of the epidermis with the endoderm and persistence of the anal opening. Both methods of wound closure were described by Abel ('02) for *Tubifex* and *Nais*.

The fusion of the epidermis with the intestinal lining is complete after twenty-four hours. The epidermal cells are no longer flattened, but appear crowded and slightly enlarged at the ends of the worms, but there are no mitotic figures among them. A reduced number of granular cells and injured muscle cells are still present in the body cavity about the end of the intestine. In the cases where the cut surface healed over without an anal opening, the blind end of the gut displaced the cicatrix and came to lie against the single outer layer of epidermal cells. In some instances there is a slight inpushing of the epidermal layer, probably due to the absence of the supporting cicatrix. The posterior movement of the blind end of the intestine is evidently brought about by contraction of the circular muscles of the gut wall, since there

is no evidence of cell proliferation in this region to add new material to the end of the intestine. The anal opening is present in all the worms after forty-eight hours' regeneration. A loose mass of granular cells remains in the body cavity dorsal to the intestine (fig. 9). The injured muscle cells, which were formerly a part of the cicatrix, have disappeared. The adjacent intestinal lining is thickened by the increased size and crowding of the endoderm cells, although there are no mitotic figures present to account for this condition. The epidermal cells of this area are considerably enlarged and contain oval, lightly staining nuclei. This is particularly true of those lying below the end of the nerve cord. Mitotic figures are present in some of these cells. In contrast to the normal condition, the epithelial layers after three days' regeneration have thickened until they consist of several layers of enlarged cells in active mitosis. They remain in this condition throughout the entire period of regeneration and furnish material for replacement of the epidermis, gut lining, and nerve cord.

The severed nerve cord does not usually extend to the wound surface. Constriction of the body-wall muscles after severing extends the end of the body posteriorly, leaving the end of the nerve cord free in the body cavity. This space is soon filled by the cells of the cicatrix which also surround the end of the nerve cord. The first evidence of nerve-cord regeneration is found after three days. A limited region of more active cell proliferation appears in the epidermis ventral to the tip of the nerve cord, and the aggregation of cells thus formed pushes the muscle layers of the body wall inward at this point. A few hours later, these cells begin to push into the body cavity and form masses of ganglion cells in the new segments. A cord of nerve fibers appears between these ganglia as they are separated by elongation of the new segments. The epidermal origin of the nervous tissue is evident (fig. 24) throughout regeneration, and the appearance of the proliferating area is the same at all times. The old ganglion cells do not participate in regeneration.

The material for regeneration of mesodermal structures in *Tubifex* is furnished by reserve cells called neoblasts. These cells appear in the wound region twenty-four hours after severing the worm. After this time they become more numerous and others are migrating posteriorly along the nerve cord, as shown in figure 7, thirty-six hours after the operation. An examination of the posterior ventral surfaces of the septa at this time and at later stages shows numerous neoblasts in process of development from peritoneal cells of these regions, as described by Kreeker ('23). These cells migrate posteriorly and accumulate at the end of the nerve cord until the region below the intestine at the posterior end is filled with a mass of dividing neoblasts and a few granular cells (fig. 9). This behavior of the neoblasts continues throughout the period of regeneration. Cell multiplication by numerous mitoses in the mass of neoblasts causes the accumulated cells to push up laterally around the sides of the intestine and to displace the remaining granular cells of the cicatrix. Ninety-six hours after the operation, the smaller cells derived from the neoblasts are already arranging themselves in plates across the ventral and lateral regions of the body cavity as the first step in formation of the new septa (fig. 10). These mesoderm cells soon push above the intestine, and the new septa are complete by the fifth day. The process continues at the tip of the worm throughout regeneration, although most of the new septa are formed during the first eighteen days. As the new segments elongate the cells composing the septa differentiate into the thin layer of muscle tissue with the covering of peritoneal cells characteristic of these structures. Posterior migration of neoblasts is greatest during the first eight days of regeneration. After this only a few of these cells can be found along the nerve cord and in the process of formation upon the septa. The continuous proliferation of smaller cells from the ventral mass of neoblasts at the tip of the worm (figs. 17, 18) apparently furnishes sufficient material for the new structures during the later stages. On the basis of these observations of neoblast activity during

posterior regeneration and similar results obtained by Kreckler ('10), neoblasts may function as organizers in the regenerating region by initiating the changes in the epithelial layers. Epithelial cells are certainly larger and more active adjacent to these cells. If this is true, the presence of the ventral mass of neoblasts at the tip of the regenerating region would account for the continued formation of new structures and likewise the failure of regeneration in absence of the neoblasts.

The longitudinal muscle layer of the body wall is continuous throughout the regenerating region except for the small area of epidermal inpushing at the end of the nerve cord. The outer cells arising in the mass of dividing neoblasts near the end of the worm attach themselves to the body wall and differentiate into the new longitudinal muscles. The muscle layers and peritoneal covering about the regenerated intestine are formed in a similar manner from neoblast derivatives that lie against the gut wall. There is some evidence of a limited movement of muscle cells from the old gut wall over the adjacent region of the new intestine, but these cells do not extend for a distance or more than one or two segments. The circular muscle layer of the body wall begins to appear near the base of the regenerating region after the longitudinal muscle layer is complete. The basal epidermal cells apparently give rise to the circular muscles without neoblast participation, since these muscles are separated from the neoblast derivatives by the differentiated longitudinal muscle layer. Migration of basal epidermal cells into the body cavity to participate in mesodermal regeneration, as reported by Wagner ('00, '06) for *Lumbriculus variegatus*, does not occur in *Tubifex*. Only the circular muscles of the body wall appear to come from this source. These results agree with the conclusions of Kreckler ('10) for *Limnodrilus* and *Tubifex* and of Scales ('27) in *Lumbriculus*.

The severed blood vessels in an operated worm immediately constrict, so that there is very little loss of blood. The blind ends of the large dorsal and ventral vessels at first extend

into the mass of cells forming the cicatrix and later into the accumulation of cells derived from the neoblasts. As regeneration proceeds, irregular lacunae filled with blood extend from the ends of the larger blood vessels and the surrounding mesoderm cells differentiate into the walls of the new vessels.

The first indications of new nephridia are paired masses of rapidly dividing cells which appear upon the ventrolateral anterior surfaces of the oldest of the new septa after six days' regeneration. They are derived from the neoblasts and push posteriorly through the wall of the septum as a convoluted cord of cells before attaching to the body wall at the site of the nephridiopore. A tube appears in the center of this cord and a nephrostome is formed at the original site of proliferation on the anterior surface of the septum. These observations upon the origin and development of the nephridia in *Tubifex* agree with those of Michel ('99) in *Allolobophora* and Meyer ('29) in *Tubifex rivulorum*.

The new segments grow to adult size in thirty-five to forty days. They then contain all the structures found in the old segments except the layer of chloragogue cells around the intestine. These cells do not appear until about thirty days after the beginning of regeneration, when a few of them are found scattered over the intestine in the most anterior new segments, as though by migration from the old body regions. The termination of these cells near the location of the cut is a convenient indication of the amount of regeneration for a considerable period after the new segments have reached adult size. Except for the absence of chloragogue cells in the new segments, the regenerated worms cannot be distinguished from freshly collected individuals and will live equally well under laboratory conditions.

X-rayed worms

No difference could be observed in the behavior of the x-rayed and control worms during the first day after severing the posterior segments, and the changes concerned in wound closure are the same in both cases. The description of this

process already given for the control worms applies equally well to the radiated worms, with exception of the cicatrix, which persists for several days longer. After twenty-four hours, however, the worms begin to show marked differences in behavior. The epithelia of the wound region do not become thickened by enlargement of the cells as in the controls and only a few mitotic figures appear in these layers. This is in direct contrast to the active proliferation of epithelial material observed in regenerating control worms. After seventy-two hours, the terminal portion of the body for a distance of approximately a segment is slightly constricted as though by muscle contraction. This process continues, forcing the end of the body away from the ends of the nerve cord and blood vessels until the condition shown in figures 3, 4, 5, and 26 is reached in about ten days. The worms retain this terminal knob without further change so long as they remain alive and there is no regeneration of the lost segments.

It is evident that the absence of epidermal proliferation in radiated worms precludes any regeneration of nervous tissue and we find that the nerve cord ends at the base of the terminal knob (fig. 26) without addition of new tissue. In radiated worms there is never any proliferation of the epidermis ventral to the cut end of the nerve cord which characterizes regenerating worms at all times.

The absence of neoblasts in x-rayed worms after twenty-four hours as compared with the large number present in the controls at this stage is the first evidence of the effect of radiation. This condition is shown in figures 6 and 7 after thirty-six hours, when the radiated worm shows none of the neoblasts that are so numerous in the control specimen. No neoblasts are ever found along the nerve cord or in the process of formation upon the septa in radiated worms examined at any time after treatment. In correlation with the absence of these cells as a source of material, mesodermal structures derived from them under normal conditions are not replaced and the radiated worms are unable to regenerate.

EFFECTS OF X-RAYS UPON THE NEOBLAST

From the preceding account it is evident that regeneration of mesodermal structures in normal worms, with exception of the circular body-wall muscles, takes place by differentiation of neoblasts or their derivatives. Since the neoblasts are not present in the x-rayed worms the failure of mesodermal regeneration would seem due to lack of material from this source. The mesodermal cells upon the posterior surfaces of the septa for several segments anterior to the cut surface were examined to determine whether there were any changes in the cells from which the neoblasts arise. No diminution in the number of these cells could be observed and they did not differ in appearance from similar cells in control worms. No stages of neoblast formation were found upon the septa of x-rayed specimens at any time after the operation. Controls at corresponding stages show many neoblasts in process of formation upon the posterior surfaces of the septa and others migrating along the nerve cord, as Kreeker ('23) described (fig. 7).

To observe the effects of radiation upon the neoblasts, normal worms were cut as in previous experiments and allowed to regenerate for forty hours. At this stage there are many neoblasts migrating along the nerve cord. These forty-hour worms were then given a thirty-minute exposure to the rays, after which they were killed at regular intervals to determine the progressive effects of radiation upon the neoblasts. The characteristic appearance of a migrating neoblast is shown in figure 19. The clear, oval nucleus contains a prominent nucleolus which is usually surrounded by a region relatively free from the granules of heavily stained chromatin lying near the periphery. The granular cytoplasm which extends along the surface of the nerve cord during migration is uniform in appearance and with the stains used appears much darker than the nucleus. After reaching the regenerating region, the neoblasts become oval in shape and soon begin active mitotic division to form the mass of smaller cells. There are no visible effects of the radiation in the

neoblasts for two hours after the exposure. After three hours, the neoblasts along the nerve cord become oval and often appear in the body cavity above the intestine, where they are never found under normal conditions. In contrast to its former condition, the nucleus now stains heavily and the cytoplasm appears clear and vesiculated. The irregular nucleolus frequently breaks into pieces and a phagocyte is often attached to the side of the floating cell (fig. 20). These changes are more apparent from five to nine hours after radiation (figs. 21, 22). There appears during this period a pronounced vacuolization in both nucleus and cytoplasm with fragmentation of the nucleolus. The attached phagocyte seems to have no part in these changes (compare figs. 21, 22), since they also occur in neoblasts that are free from these cells. The nucleus begins to fragment about thirteen hours after radiation, and this, with the increased vacuolization, leads to disintegration of the neoblast a few hours later (fig. 23). Only an occasional degenerating cell is present at eighteen hours, and at twenty-two hours the neoblasts have completely disappeared. A comparison of the radiated and control neoblasts thirteen hours after treatment (figs. 23 and 19) shows the extent of these changes. If a phagocyte is attached to the neoblast, the cytoplasm first disappears and the dark nuclear material remains for a time surrounded by the phagocyte before disintegrating. The mass of neoblasts that has collected at the end of the nerve cord near the cut surface before radiation shows the same series of degenerative changes within the cells and disappears completely. There is no further change, except the formation of an anal knob by rearrangement of the terminal tissues, as previously described.

EFFECT OF X-RAYS UPON CELL DIVISION

In addition to the effect upon the neoblasts and the peritoneal cells from which they arise, it appears that radiation also brings about changes in other cells which prevent participation of these elements in regeneration. Replacement

of the nerve cord and epidermis is dependent upon cell multiplication in the epidermis. It has been pointed out that the number of mitotic figures is so greatly reduced in this layer of the x-rayed worms as to be almost negligible. A comparison of the number of mitoses counted in the epidermis of the terminal region in x-rayed and in control worms makes it evident that only a small amount of new tissue could be formed by this means. The destructive effects of radiation upon the cell during mitosis and the subsequent irregularities in cell division have been observed by Richards ('14), Packard

TABLE 7

Comparison of the mitotic figures in the epidermis of the regenerating region in the x-rayed and control worms

X-RAYED 30 MINUTES	TWENTY TO THIRTY POSTERIOR SEGMENTS REMOVED								
Number of worms	2	3	3	2	2	2	2	2	2
Hours of regeneration	24	48	72	96	120	144	168	216	288
Number of mitotic figures	0	0	4	0	0	1	6	10	0
CONTROLS	TWENTY TO THIRTY POSTERIOR SEGMENTS REMOVED								
Number of worms	2	3	3	2	2	2	2	2	2
Hours of regeneration	24	48	72	96	120	144	158	216	288
Number of mitotic figures	0	5	16	29	33	53	101	136	107

('16), Alberti and Politzer ('23, '24), and many other investigators. After radiation of vegetative epidermal cells of urodeles, Alberti and Politzer found a reduced number of irregular mitoses showing pycnosis and fragmentation of the chromosomes. Similar results were observed in the interstitial cells of radiated *Pelmatohydra* by Zawarzin and Strelin ('28). It is well known that overexposure to x-rays is injurious to the basal epidermal cells in man. In the epidermis of annelids, cell replacement occurs largely by division of the basal cells which remain in an embryonic condition. Wagner ('00, '06) called these cells 'dermoblasts' in *Lumbriculus*, and mentions their rapid division during regeneration. The

chromosomes that appear in the occasional mitotic figures in the epidermis of x-rayed worms are clumped together so that it is impossible to observe fragmentation or irregular divisions as reported in other forms. The small size of these cells, together with the thickness of the sections and the stains used, precludes observations upon changes in the appearance of the protoplasm. In view of the known differential effect of x-rays upon embryonic tissues, it is not unlikely that the basal cells of the epidermis are most affected by radiation, and if not destroyed are at least inhibited from division. Hence there is no material for regeneration of the epidermis and nerve cord and the anal knob is formed by rearrangement of the old tissues.

DISCUSSION

The cycle of neoblast activity can be divided into three periods. The first or growth period of about fifteen hours is passed entirely upon the septum and represents the time necessary for growth of peritoneal cells into the characteristic neoblasts as they leave the septa. This is followed by the period of migration lasting a few hours and during which there are no changes except those due to the amoeboid movements of migration. Upon reaching the wound region, the neoblasts cease moving and enter upon the period of proliferation and differentiation which continues throughout regeneration. Each stage of neoblast activity is thus characterized by definite reactions.

The question of continuity presents itself because neoblasts as such are not present continuously upon the posterior surfaces of the septa. They may be defined as migratory stages of undifferentiated cells arising, after injury to the worms, by growth from peritoneal cells that one might call 'potential neoblasts.' Neoblasts can only be distinguished from the surrounding peritoneal cells after the onset of this growth. When they have thus arisen they must be regarded as undifferentiated cells, since they divide in the regenerating region into smaller cells from which new mesodermal structures are

formed, including new peritoneal cells from which neoblasts may later arise upon the posterior faces of new septa. There is, therefore, no reserve of neoblasts present at all times, as the 'formative cells' of planarians (Curtis, '28), unless the peritoneal cells can be so regarded. These latter constitute a special tissue, though not a tissue that is highly differentiated. One might regard the growth of peritoneal cells to form neoblasts as a dedifferentiation by which undifferentiated neoblasts arise from specialized cells. It seems more reasonable to regard these cells of the peritoneum as cells without much differentiation which under appropriate stimulation can grow, like egg cells, by addition of stored products in their cytoplasm and correlated changes in their nuclei and so become neoblasts. The basal cells of the ectodermal and epidermal epithelia are more obviously a reserve of undifferentiated cells that functions in the growth of these layers throughout life, and one that is stimulated to more rapid multiplication during regeneration. The neoblasts must be regarded as undifferentiated cells because of their ability to differentiate into several kinds of mesodermal tissues. The smaller cells from which they arise upon the septa may be regarded as a reserve of inactive reparative material.

If the destruction of migrating neoblasts by an x-ray exposure, which has no apparent effect upon the surrounding tissues other than the check upon mitosis already mentioned, is to be taken as additional evidence for the undifferentiated nature of these cells, one would suppose that the peritoneal cells from which the neoblasts arise would be even more susceptible to radiation. Yet there are no structural changes to be observed in these peritoneal cells following exposures to the rays that kill all the neoblasts and destroy the regenerative power of the ectodermal and mesodermal epithelia. The failure to give rise to neoblasts is the only evidence that the peritoneal cells are affected by the rays.

The formation of new epithelial tissue from the old epidermal and endodermal layers at the onset of regeneration in *Tubifex* involves little activity on the part of the differenti-

ated cells in these regions. Wagner ('06), in *Lumbriculus*, mentions the active proliferation of smaller basal cells which he calls 'dermoblasts' and a similar relationship seems to exist in *Tubifex*. Proliferation of new material apparently does not involve participation of the functional epidermal cells, but the assumption of active division by the smaller basal epidermal cells which have remained in a relatively undifferentiated condition. In contrast to this condition, these epithelial layers in the x-rayed worms show only an occasional mitotic figure. While no changes in appearance of these basal epithelial cells in radiated worms could be observed, the exposure affects them in such a manner that they do not divide and furnish the new material.

After the preliminary exposures, a latent period of twenty-four hours was allowed, but a shorter time would have been equally effective. Only a few hours are necessary for the effects to become apparent as visible changes in structure of the neoblasts, and it is probable that no longer time is required for the peritoneal cells to become affected in the functional manner above described. As the effect of the rays upon regeneration is the same when the worms are severed at the time of exposure, it is evident that no allowance need be made for a latent period in such experiments with *Tubifex*.

The degenerative changes in migrating neoblasts after radiation as recorded in this paper may be regarded as only death stages of these cells like those that would have occurred following exposure to other lethal agencies. To determine effects of radiation other than mere killing, it would be necessary to keep the neoblasts alive and observe changes in the living cells induced by shorter exposures to the rays. It might thus be possible to observe the regions within the cells that are first affected by the treatment and the progress of such changes in response to increasing exposures. What has been observed is that exposure to x-rays renders the animal incapable of regeneration. The migrating neoblasts are destroyed and further neoblast formation from peritoneal cells is in some manner inhibited. One can say that the power

of regeneration is lost because the cellular material is thus lacking in the same manner as in radiated *Planaria*, *Tubularia*, *Hydra*, and *Clavelina*, in which failure of regeneration is correlated with destruction of reserves of embryonic cells. But the mechanism of this destruction is unknown. It is presumably by direct action upon these cells, although it may be that other parts are the ones directly affected and that the effects seen in the neoblasts are really secondary.

The rapid disintegration of neoblasts after radiation and the absence of division in these cells during migration prevent observation of radiation effects upon the chromosomes. Destruction of these cells results from cytoplasmic changes, if one may judge from the fact that the cytoplasm is first to show changes, as indicated by vacuolization and reversal of normal staining capacity. Similar changes within cells after heavy radiation have been reported by many investigators, and they invariably precede disintegration. Whether this is due to chemical changes in the compounds within the cell instituted by direct action of the rays, as Schwarz ('03) and many later investigators have maintained, or follows alternation of the body fluids surrounding the cells, or results from destructive changes in the colloidal organization of the protoplasm as postulated by some recent investigators, like Fernau ('25), cannot be determined from the material at hand. My observations show only that migrating neoblasts can be destroyed by the radiation and that additional neoblasts are not formed in worms so treated. The failure of mesodermal regeneration can be accounted for by this loss of necessary cellular material.

It is possible to distinguish clearly between the processes involved in wound healing and regeneration in *Tubifex*. In normal specimens the wound healing takes place during the first twenty-four hours after severing the worms and before the neoblasts have arrived at the cut end or the epidermal cells have enlarged prior to beginning regeneration. The presence of neoblasts and their derivatives and of mitoses in the thickened epidermal layer differentiates the period

of regeneration from that of wound healing which takes place before these characteristics become evident. The separation of the two processes is also evident in radiated worms which heal the wound but do not regenerate.

The use of x-rays as a technique (Curtis, '28) is further illustrated by these results in *Tubifex*. By utilizing the known differential effect of the rays upon embryonic tissues, certain regions may be altered without apparent injury to the remainder of the organism. Such a technique may be applicable in investigations of embryonic development dealing with parts that exhibit various degrees of differentiation and hence might have various susceptibilities to such radiations. For example, the cells derived from the neoblasts must pass through a series of stages as they approach complete differentiation into the new structures and at each step they will become progressively less embryonic in character. On the basis of these changes it should be possible by use of x-rays to demonstrate the progress of differentiation in relation to the time at which the cells become more resistant to exposure. Beginning with an exposure sufficient to destroy migrating neoblasts, the differentiating cells should withstand increasing exposures leading to the final stage in which they are least susceptible to the rays. In worms radiated forty to fifty hours after posterior regeneration had begun, the neoblast derivatives forming the new septa are least affected and remain for a time after all the neoblasts have disappeared, as would be expected from their degree of differentiation.

While considerable has been learned regarding the differential susceptibility of tissues, which is the foundation for use of x-rays as a technique of investigation, we are still uncertain concerning the changes by which these effects are brought about. Schwarz ('03) was among the first to observe the effects of radiation upon protoplasm. He found that lecithin of egg yolk was decomposed by exposure to radium emanations and upon this basis advanced the hypothesis that radiation effects upon cells were due to similar alterations of

the cell contents. O. Hertwig ('11), from observations of the development of radiated frog eggs fertilized with normal and radiated sperm, concluded that only the chromatin within the nucleus is affected by radium emanations so that abnormalities which appear during development arise from this source. It was suggested by Richards ('14), after treatment of *Planorbis* eggs with x-rays, and by Packard ('14) from studies of *Arbacia* eggs exposed to radium emanations, that irregularities in development were not brought about by direct action of the emanations, but indirectly by effects of the rays in increasing the activity of autolytic cell enzymes which bring about the observed changes in the chromatin and protoplasm. More recently, attempts have been made to explain x-ray effects as due to alterations in the colloidal structure of protoplasm similar to those observed after radiation of inorganic preparations. Fernau ('25) considers these theories with others that have been advanced and concludes that none of these factors will explain the observed effects of x-rays on cells. The conclusions of Schwarz, O. Hertwig, and Packard are based upon observations of eggs detached from the parent organisms. In the case of *Tubifex*, one must admit the further possibility that the rays may have affected the body fluids surrounding the neoblasts in a manner to induce the observed destruction of these cells. It is well known that radiation brings about changes in compounds, of which the decomposition of lecithin already mentioned is an example. The methods of technique used in these experiments would give no indication of such changes if they had occurred. The introduction of migrating neoblasts from normal regenerating worms into radiated worms with subsequent observations of their behavior would test this possibility, but I have not been successful to date in thus transferring neoblasts.

SUMMARY

Posterior regeneration in *Tubifex tubifex* occurs readily from different levels of the body.

The level of the cut within the segment has no effect upon this regeneration.

The epidermis regenerates by proliferation of basal cells within the old epidermal layer.

The new nervous tissue regenerates by proliferation from a limited area of the epidermis near the cut end of the old nerve cord.

The lining of the new intestine, with the exception of a short proctodaeum, regenerates by proliferation of basal cells in the old endodermal epithelium.

The neoblasts arise from peritoneal cells upon the posterior faces of the septa in segments adjacent to the wound area. They migrate posteriorly along the ventral body wall and nerve cord, and accumulate at the posterior end of the worm, where they divide and differentiate into new mesodermal tissues.

The longitudinal muscles of the body wall and those of the blood vessels and intestinal walls in the regenerating region are formed by small cells which arise by division of these neoblasts; the circular muscles of the body wall apparently regenerate from basal epidermal cells.

There is no regeneration when the posterior end is removed from worms that have been exposed to x-rays. Only a small anal knob is formed by rearrangement of tissues in the terminal region, with no trace of the structures so conspicuous in normal regeneration. No neoblasts appear in such radiated worms.

In worms allowed to regenerate until the neoblasts are migrating along the nerve cord and then exposed to x-rays, the neoblasts in all stages of formation, migration, and posterior accumulation are killed within twenty-four hours and no additional neoblasts are formed by the peritoneum.

This inhibition of regeneration and the loss of power to form neoblasts is continued throughout the period of each

experiment and in one experiment for as long as 147 days. The worms thus behaved as though 'castrated' against regeneration.

CONCLUSIONS

The inhibition of regeneration from the epidermis and the intestinal epithelium can be explained by the inhibition in radiated *Tubifex* of the mitotic divisions that occur in the basal cells of these epithelial layers during normal regeneration. In the absence of such cell multiplication no regeneration can occur whether or not the organismal factors that may influence regeneration persist.

The inhibition of regeneration from neoblasts can be explained by the destruction of these cells in all stages of growth, migration, and posterior accumulation and by the inhibition of their formation from cells of the peritoneum.

The observations recorded furnish no basis for conclusions regarding the specific effects of the rays upon the epithelial cells or the neoblasts, nor their specific effect upon peritoneal cells from which neoblasts would normally be produced. The vacuolization of nuclei and cytoplasm in neoblasts, following radiation, are no more significant than the necrotic changes that might follow the action of any lethal reagent. No cytological changes are apparent in the material as a result of radiation, but something takes place in the worm that checks mitosis in the basal epithelial cells and prevents the formation of neoblasts from peritoneal cells following the stimulation of removing the posterior end.

No reserve of undifferentiated cells is normally present in *Tubifex*, unless the peritoneum from which the neoblasts arise and the basal cells of body and gut epithelia can be so interpreted.

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PLATES

ABBREVIATIONS

a, anal opening
ant, anterior
ak, anal knob
b.v., blood vessel
chl, chloragogue layer
int, intestinal lining
epi, epidermis
g, ganglion cells
gnc, granular cells
int, cavity of intestine

mus, muscle layer
mus.c, muscle cells
n, ventral nerve cord
nco, neoblast
nu, nucleus
phag, phagocyte
post, posterior
s, septum
v, vacuole
x-x, plane of cut

PLATE 1

EXPLANATION OF FIGURES

- 1 X-rayed worm, seven days after removal of posterior segments. Location of the cut is indicated by the line in all figures. $\times 25$.
- 2 Control worm, seven days after removal of posterior segments. $\times 25$.
- 3 X-rayed and control worms, fourteen days after removal of posterior segments. $\times 25$.
- 4 X-rayed and control worms, twenty one days after removal of posterior segments. $\times 25$.
- 5 X-rayed and control worms, thirty days after removal of posterior segments. $\times 25$.
- 6 Photograph of sagittal section of x-rayed worm, thirty-six hours after removal of posterior segments. $\times 130$.
- 7 Photograph of sagittal section of control worm, thirty six hours after removal of posterior segments. $\times 150$.

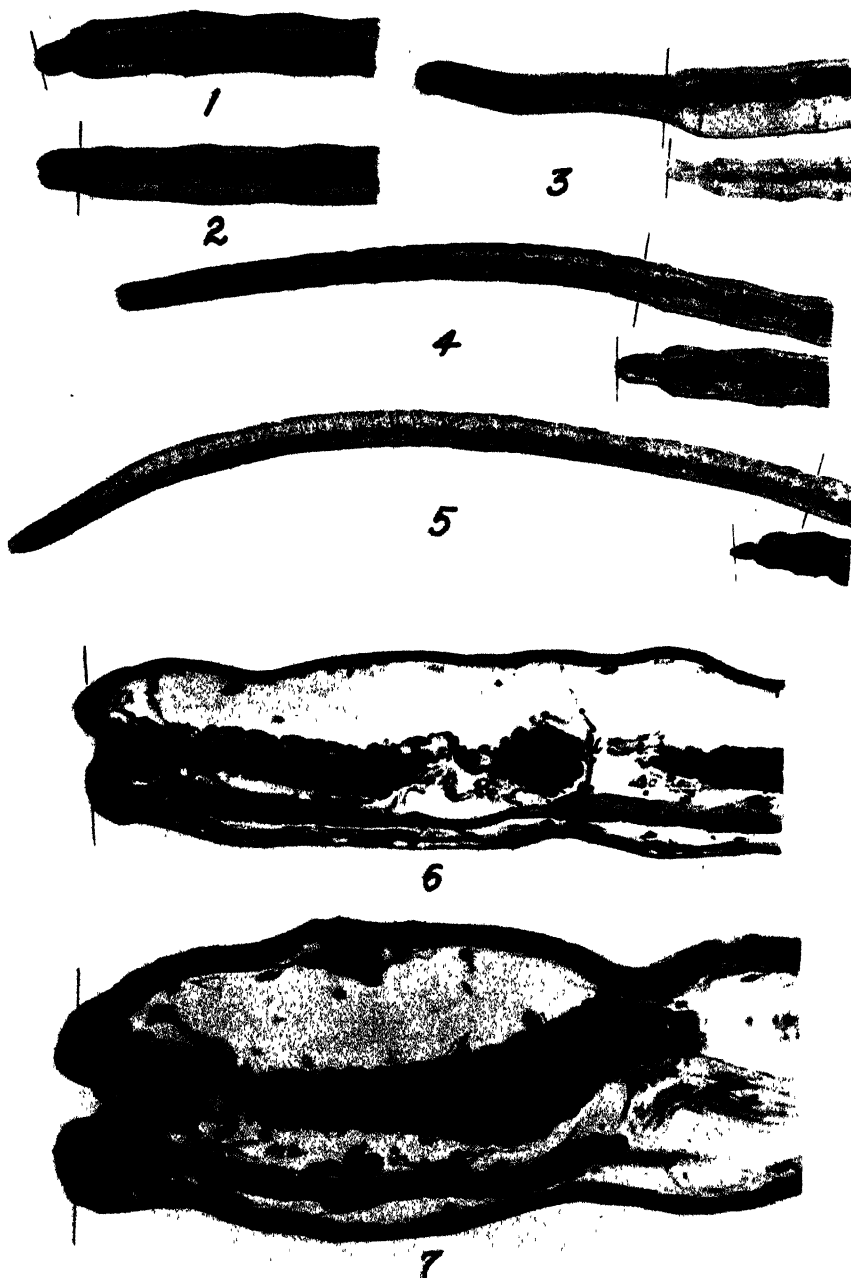


PLATE 2

EXPLANATION OF FIGURES

8 Sagittal section of x-rayed worm, forty-eight hours after removal of posterior segments. $\times 138$.

9 Sagittal section of control worm, forty-eight hours after removal of posterior segments. $\times 138$.

10 Sagittal section of control worm, seventy-two hours after removal of posterior segments. $\times 138$.

11 Sagittal section of control worm, ninety-six hours after removal of posterior segments. $\times 138$.

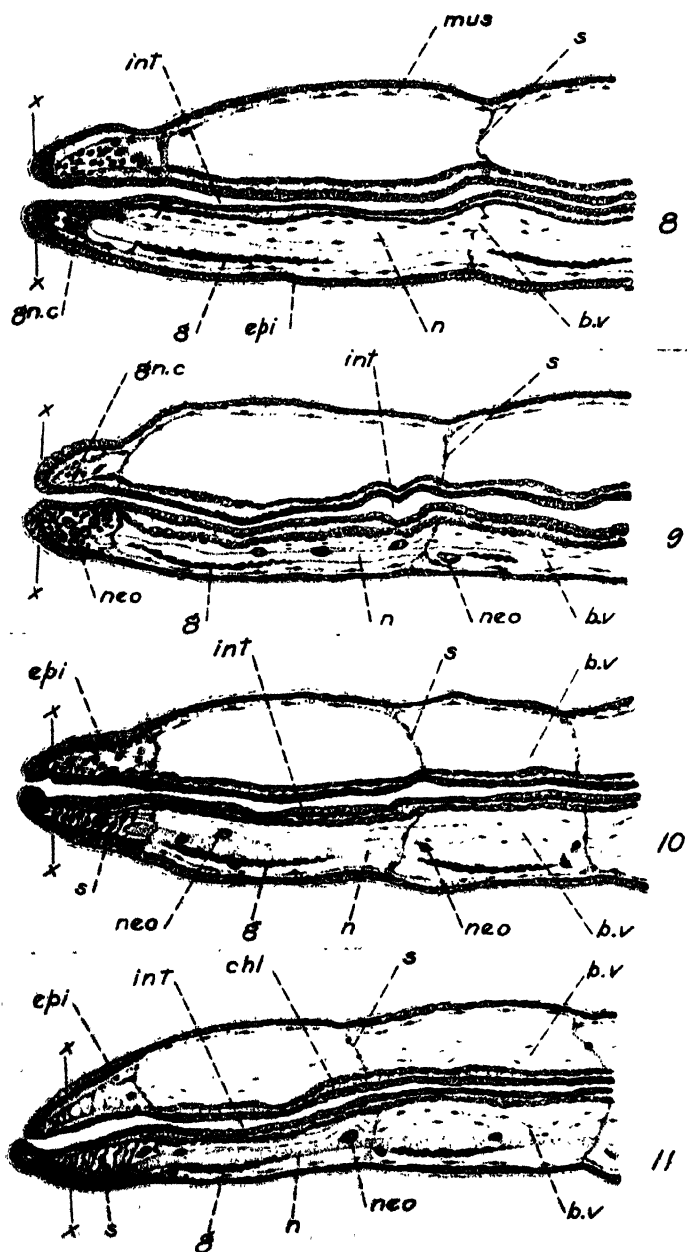


PLATE 3

EXPLANATION OF FIGURES

12 Sagittal section of x-rayed worm, 120 hours after removal of posterior segments. $\times 138$.

13 Sagittal section of control worm, 120 hours after removal of posterior segments. $\times 138$.

14 Sagittal section of control worm, 144 hours after removal of posterior segments. $\times 138$.

15 Sagittal section of control worm, nine days after removal of posterior segments. $\times 138$.

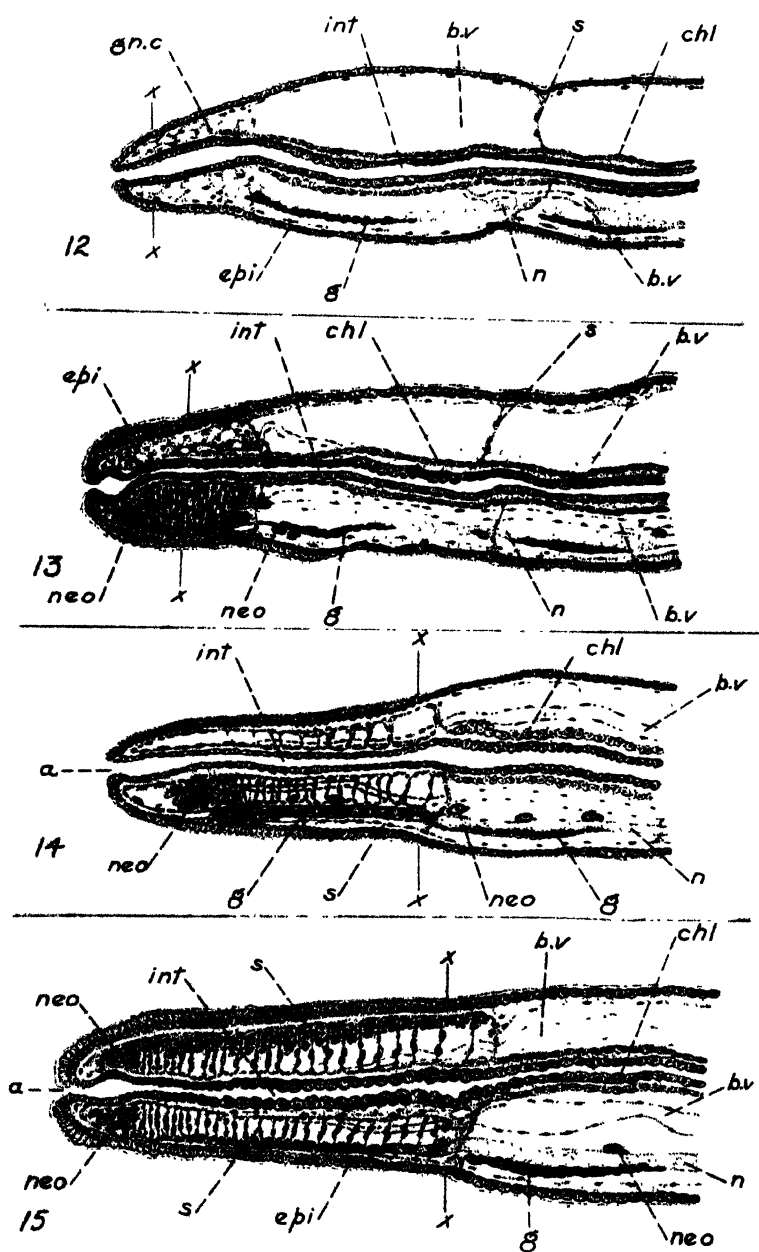


PLATE 4

EXPLANATION OF FIGURES

16 Sagittal section of x-rayed worm, twelve days after removal of posterior segments. $\times 138$.

17 Sagittal section of control worm, twelve days after removal of posterior segments. $\times 138$.

18 Sagittal section of control worm, eighteen days after removal of posterior segments. $\times 68$.

19 Normal neoblast when migrating along nerve cord. $\times 1550$.

20 Degenerating neoblast, with phagocyte attached, three hours after thirty minutes' exposure to x-rays. $\times 1550$.

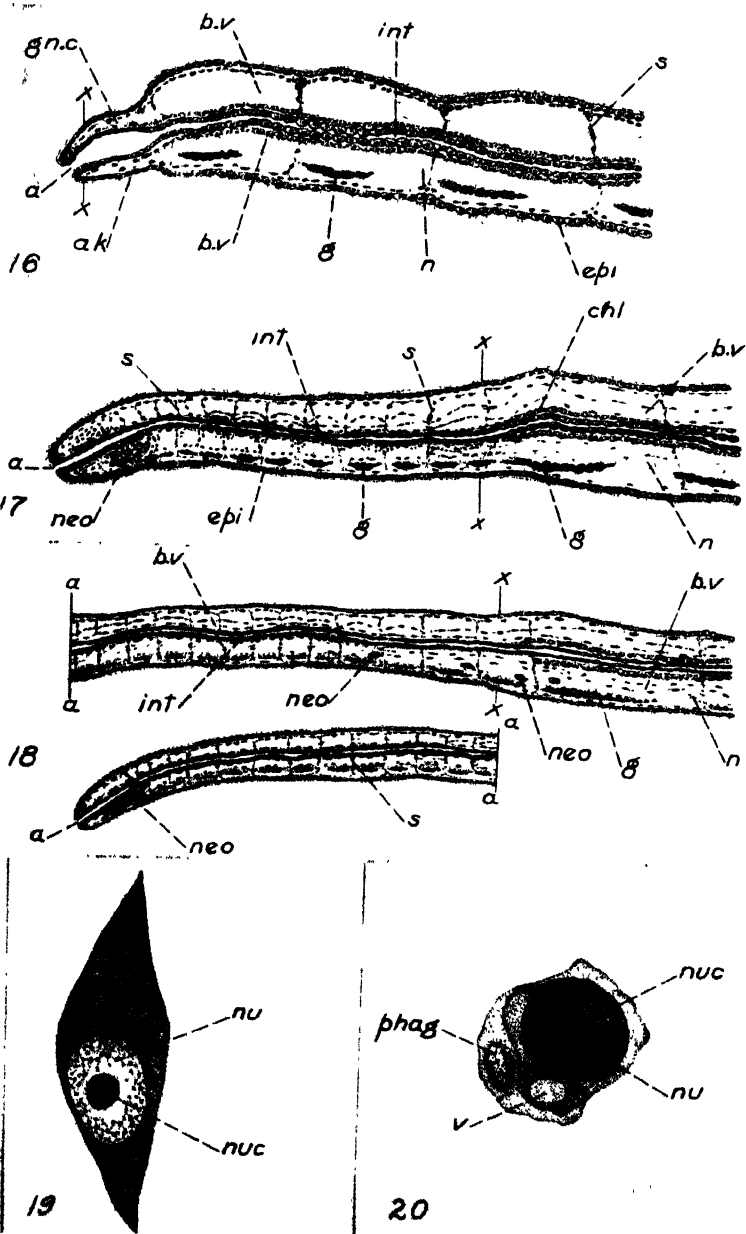


PLATE 5

EXPLANATION OF FIGURES

21 Degenerating neoblast, with phagocyte attached, five hours after thirty minutes' exposure to x-rays. $\times 1550$.

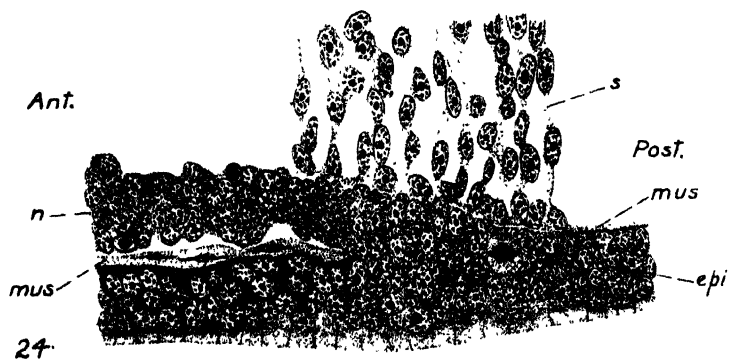
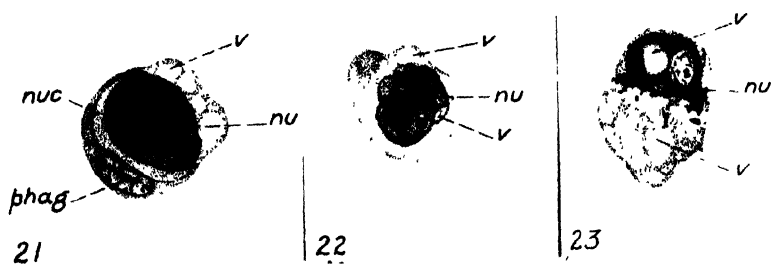
22 Degenerating neoblast, nine hours after thirty minutes' exposure to x-rays. $\times 1550$.

23 Degenerating neoblast, thirteen hours after thirty minutes' exposure to x-rays. $\times 1550$.

24 Sagittal section of control worm, nine days after removal of posterior segments. Formation of the new nerve cord and septa is shown. $\times 775$.

25 Photograph of sagittal section of control worm, nine days after removal of posterior segments. $\times 145$.

26 Photograph of sagittal section of x-rayed worm, twelve days after removal of posterior segments. $\times 170$.



COMMENTS ON THE ORIGIN OF THE MIDGUT IN INSECTS

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TWO PLATES (NINETEEN FIGURES)

AUTHOR'S ABSTRACT

Reviews the question of the origin of the midgut of insects with its diverse appearances and interpretations. Without doubt the midgut epithelium in some insects is derived from the lower layer, but in other insects from the tips of the stomodaeal and proctodaeal invaginations. The question of its origin seems to be "a function of the position within the whole," and best understood from a standpoint of the time of determination of the parts involved. Maps illustrating the prospective significances of the principal types are included and discussed.

There are a number of ways in which the epithelial wall of the midgut is formed in insects. In some (i.e., the cockroach) an elongated gastrular furrow is formed (fig. 3), the edges of which, coming together, cut off a tubular 'lower layer' (figs. 4 and 5), the middle strand breaking down except at the extremities. The cells of the broken-down median strand contribute to the formation of the blood cells in part and disintegrate in part, whereas the extremities are carried in on the tips of the stomodaeal and proctodaeal invaginations and by subsequent growth give rise to the midgut walls (figs. 6 to 8, *end.*). In many insects, instead of a gastrular furrow resulting in a tubular lower layer, the process is speeded up, so to speak, and an unilaminar lower layer is formed (figs. 9 to 14). During the formation of the nerve cord the median strand of the lower layer usually breaks down as in the first case, though there are numerous examples in which it contributes to the midgut directly (*Periplaneta*, *Donacia*, etc.). In some cases the extremities are left intact and, being carried in on the tips of the stomodaeal and proctodaeal invaginations, give rise to the wall of the midgut much as in the cockroach (*Endromis* (*Lepidoptera*)), as figured and

described by Schwangart). In many cases, however, this median strand is totally broken down and no cells are carried in on the tips of the stomodaeal and proctodaeal invaginations (fig. 14); later, then, there is a proliferation of cells at the ventrolateral corners of each of these invaginations, and from these cells the anlage of the midgut is formed (figs. 15 and 16 to 19, *m.g.*). In *Catocala* this last is the case (Hirschler, '05), but here the cells of the broken-down median strand contribute to the formation of the midgut. The latter type led Heymons and later others (Schwartz, Hirschler, etc.) to claim that the midgut in these forms is ectodermal in contradistinction to endodermal in the cockroach, etc.

Johannsen ('29), working on *Diacrisia* (Lepidoptera), attempted to bring these diverse views together by an idea of 'latent endodermal cells,' as Heider had suggested earlier. At his suggestion the present author took up a further study of this point, using *Diacrisia virginica* and *latipennis*, *Estigmene acraea* and *congrua*, and *Isia isabella* (related genera of Arctiidae). An abundance of material was obtained at this point of embryonic development, and the results show that in this case the midgut is formed similar to the way shown by Schwartz ('99) for *Lasiocampa*. At no point was there any positive evidence that the 'blood cells' derived from the broken-down median strand contributed to the formation of this epithelial wall such as Hirschler ('05) has reported for *Catocala* (Noctuidae).

A series of photomicrographs (figs. 16 to 19) illustrates this point. In figure 16 we have a section of the stomodaeum without any trace of midgut anlage, but in figures 17 to 19 we see how this develops. Later these strands continue to grow until they meet and fuse with those growing forward from the proctodaeum, close across ventrally by growth, and then slightly later close over dorsally after the rotation of the embryo and just prior to the closure of the body wall.

These figures show fundamentally the same developmental history that has been described quite a number of times in diverse insect groups, and so it seems safe to assume that

the facts are as stated above. Are we to conclude that the midgut in some insects is an endodermal derivative and in others an ectodermal derivative? Or can we have recourse to the idea of 'latent endodermal cells'? In one sense, though probably not the original one, I believe we can accept this latter view.

If we take a cockroach embryo and, working backward, map the regions of the germ band, as Johannsen has done, we obtain a map of 'prospective significances' as shown in figure 1. If, on the other hand, we do this for *Diacrisia*, etc., we obtain a modification as in figure 2, where the white circles (*st.* and *pr.*) indicate the tips of the stomodaeal and proctodaeal invaginations. Assuming a strictly morphological concept of germ-layer formation, we will get one of two interpretations. Either there is no endoderm (or the endoderm is entirely broken down, i.e., the median strand), or else the tips of the stomodaeal and proctodaeal invaginations represent 'latent endoderm cells,' in which case the circles on our map might be labeled *end.* instead of *st.* and *pr.* (fig. 2). Other cases, such as when the median strand is not broken down, but contributes directly to the midgut epithelium, or where it is broken down and partially reassembled, would give somewhat different maps of 'prospective significance.'

We must be cautious, however, against placing too much faith on such maps. They tell us nothing more than that in *normal* development this or that part of the germ band will come to form this or that particular part of the embryo. They do not imply that they can form nothing else, i.e., that they are already 'determined.' So the experimental embryologists, notably the workers on Amphibia, distinguish between 'prospective significance' and 'prospective potency' and bring in the idea of 'time of determination,' etc. At this point we may compare the elaborately worked-out map of prospective significances of the parts of the Triton egg given us by Vogt with the results of the transplantation experiments of Spemann et al. Within the last few years experimental work has been begun on insect embryos, notably by

Seidel, but the technique is still insufficiently developed to settle the point at issue here.

Pending pertinent experimental data, we are forced, it seems to me, to one of two conclusions: either the midgut, despite its final similarity within individual orders of insects, may arise in some cases as an endodermal derivative and in other cases as an ectodermal derivative; or else we must look upon the determination of the midgut as a purely physiological process¹; to borrow Driesch's terminology, "as a function of the position within the whole." That is, that whatever cells in each particular case happen to be appropriately located at the time when the endodermal strands are due to be formed, those cells will be 'determined' to form the midgut anlage, and subsequently differentiate into the structure typical of midgut epithelium. The question is purely a matter of the ~~time~~ of determination of the parts involved, and the latter interpretation seems to me much superior to the former in the light of the great diversity shown by closely related insects, and from the standpoint of modern views of developmental processes in general. There is a somewhat analogous anomaly in the formation of the notochord in the chordate series.

Nevertheless, it is still useful at times to make these 'promorphological' maps of prospective significances for the understanding of the developmental history of various insects, despite the differences from one form to the next, provided we hold in mind that they indicate nothing as to the potencies or time of determination of the various parts indicated. Needless to add, they form an indispensable basis for experimental work.

¹Hirschler ('05) has said, "My observations, as well as those of Tchouproff, show that the different aspects thruout the Pterygotes, and finally on the one hand the views of Heymons, Czerki and others who hold that the midgut is a truly ectodermal derivative, and on the other hand those of Noack, Dickel, Schwangart and others who maintain that it is a truly endodermal derivative, may be united in a certain sense" (p. 810).

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ABBREVIATIONS

<i>am.</i> , amnion	<i>g.b.</i> , germ band
<i>ant.</i> , anterior end of embryo	<i>mds.</i> , median strand
<i>b.c.</i> , 'blood cells'	<i>mcs.</i> , mesoderm
<i>b.w.</i> , lateral body wall	<i>m.g.</i> , midgut anlage
<i>ect.</i> , ectoderm	<i>n.c.</i> , neural cord
<i>end.</i> , endoderm	<i>pr.</i> , proctodaeum
<i>f.end.</i> , freed endoderm cells from median strand	<i>s.b.</i> , suboesophageal body (glandular)
<i>f.mcs.</i> , freed mesoderm cells, the so-called 'blood cells'	<i>scr.</i> , serosa
	<i>st.</i> , stomodaeum
	<i>t.st.</i> , tip of stomodaeal invagination

PLATE 1

EXPLANATION OF FIGURES

1 Map of the prospective significances of the egg of a primitive type such as the cockroach spread out flat in a single plane. The dark spots on the mesial margin of the ectoderm indicate the neuroblastic centers; similarly, the distal margin of the mesodermic strips might be shown as distinct to indicate its contribution to the blood cells. (After Johannsen, '29.)

2 Same for Diacrisia and allies. The white circles represent the tips of the stomodaeal and proctodaeal invaginations without any implication as to germ-layer derivation.

3 Diagrammatic cross-section through the germ-band region as the gastrular furrow is beginning to form. Ventral side uppermost. (After Johannsen, '29.)

4 Same, somewhat later. (After Johannsen, '29.)

5 Same later, showing the formation of the neural groove and liberation of the cells of the median strand and of the blood cells. (After Johannsen, '29.)

6 to 8 Longitudinal series of one end of the same type illustrating the origin of the endoderm. Figures 6 and 7 correspond to stages between those represented by figures 4 and 5, and figure 8 represents about the same stage as figure 5. (Figures 6 and 7 after Johannsen, '29.)

9 to 12 Series of diagrammatic cross-sections illustrating germ-layer formation in Diacrisia and its allies.

13 to 15 Series of diagrammatic longitudinal sections of one end of a Diacrisia embryo, showing the origin of the midgut epithelium without any contribution from the 'lower layer.'

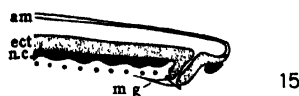
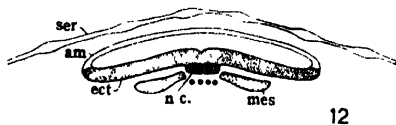
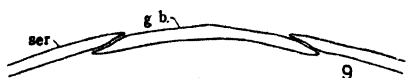
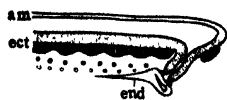
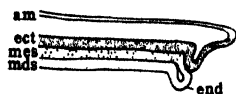
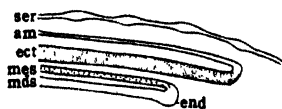
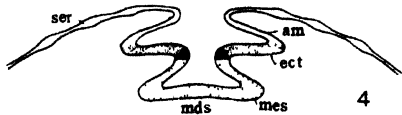
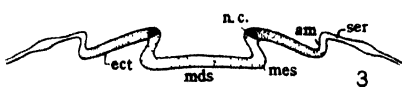
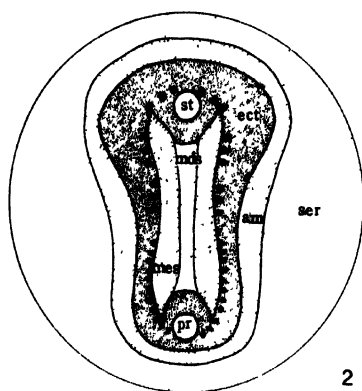
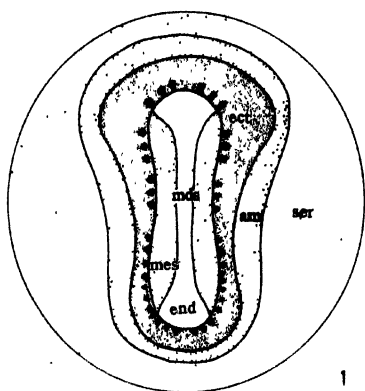


PLATE 2

EXPLANATION OF FIGURES

Photomicrographs of sections cut at 5μ and stained with Mayer's haematoxylin and counterstained with erythrosin. Photographed with the use of a Wratten green filter. $\times 310$.

16 Longitudinal section through the stomodaeal invagination of an embryo of *Estigmene aceraca* about fifty-eight hours old. The section is slightly oblique and so does not show the opening to the outside, but it does show quite clearly the unilaminar nature of the end of the stomodaeal invagination. No indications of the midgut anlage are to be found in this or adjacent sections. The sub-oesophageal body (glandular) lies just below the stomodaeal invagination, but is always distinct from it.

17 Longitudinal section through the stomodaeal invagination of an embryo of *Diacrisia latipennis* about sixty-one hours old. Shows the unilaminar nature of the end of the stomodaeal invagination with the first beginnings of midgut anlage. Due to the focal depth involved, not all points of interest are clear, but the initial cell multiplication at the ventral part of this membrane is evident.

18 Longitudinal section through the stomodaeal invagination of another embryo of the same lot as figure 17, showing a slightly later stage in the formation of the midgut anlage. The blood cells seen in the neighborhood may possibly contribute to the formation of the midgut as Hirschler has reported for *Catocala*, but the present author thinks not.

19 Another embryo of the same lot as the last two, but cut in a diagonally dorsoventral direction and somewhat obliquely. It is somewhat more advanced in development, and shows the limiting membrane unilaminar but swollen throughout most of its extent, and with the considerably developed midgut anlage growing ventrolaterally.



POLYPLOIDY AND METAPHASE PATTERNS

EDMUND B. WILSON

TWENTY-FOUR TEXT FIGURES AND ONE HEMIOTYPE PLATE

AUTHOR'S ABSTRACT

A cyst of tetraploid first spermatocyte metaphases is described in the coreid hemipter *Archimerus alternatus* (Say), all other divisions in the testis being of normal diploid constitution. The striking fact is that in spite of the doubling of their number the chromosomes closely follow the group pattern characteristic of the corresponding normal divisions. In the latter the first metaphase always shows a ring of six autosome bivalents with a single m-chromosome bivalent at its center and a single univalent X-chromosome lying outside the ring (as in coreids generally). In corresponding tetraploids the numbers are respectively 12, 2, and 2. Three additional interesting features of the tetraploids are: 1) the fact that the two m-bivalents are always lined up end to end to form an axial quadrivalent chain, 2) that although two X-chromosomes are present (as in the normal female), they are never united to form a bivalent as in that sex; and, 3) that in the prophase (of which a few are present in the cyst), at least one pycnotic X, or chromosome nucleolus, is present.

A critical discussion is offered of the general problem of the mechanism of chromosome movements and groupings, together with a review of recent literature. The conclusion is urged that the chromosomes themselves play an active and important part in these processes, and the possible genetic relations between chromosomes and spindle substance are discussed.

It has long been known that definite arrangements or group patterns are often displayed by the chromosomes during the metaphases of mitosis, but apart from a few attempts to explain them on a simple mechanical basis their determination offers a problem that still awaits a solution. My attention was once more directed to this subject by the accidental discovery of a single tetraploid cyst of first spermatocyte metaphases in the coreid hemipter *Archimerus alternatus* (Say).¹ A series of twenty-one consecutive sections through this cyst shows that, with exception of a few late prophase at one side, all the cells are at or very near the metaphase, and that in all, the number of chromosomes is double the

¹ For the normal cytology of this species see Wilson, '05.

normal, i.e., sixteen instead of the normal eight.² The point of especial interest in these tetraploids lies in the fact, here first made known I think, that in spite of the doubled number of chromosomes they everywhere follow more or less closely the same differentiated pattern as that characteristic of the corresponding normal diploid mitoses. This surprising fact seemed to me worthy of close attention because *Archimerus* is cytologically a typical representative of the Coreidae, a group of Hemiptera heteroptera long noted for the extreme clearness with which individual differences among the chromosomes are displayed and for the definite and constant groupings which they assume during the spermatocyte metaphases.³ In this respect the Coreidae, together with the Notonectidae, Gelastocoridae, and Reduviidae⁴ are of exceptional interest for the study of chromosome movements and groupings. Though the main facts in the normal mitoses have long been familiar, a brief review will facilitate this discussion.

In *Archimerus*, as in all other coreids thus far examined cytologically since Paulmier's pioneer studies on *Anasa* ('99), there are three visibly different classes of chromosomes, each showing its own distinctive type of behavior. These are: a) the 'ordinary' chromosomes or 'autosomes'; b) the 'm-chromosomes,' a single pair, characterized by their very

* This condition is confined to a single cyst, all other divisions in the testis being normal, I conclude, accordingly, that it must have arisen in a single spermatogonium in which the original diploid number was doubled (by monocentric mitosis or the like) the tetraploid condition thus produced having been thereafter perpetuated by normal bipolar mitosis. I conclude further that (with certain exceptions described in the text) normal synapsis must have taken place in the tetraploid cyst, so as to produce the diploid number of bivalents. This is indicated by the distinct tetrad structure visible in some of the autosomes, and is demonstrated in case of the m-quadrivalent. This interpretation is in harmony with a number of earlier observers, including, for instance, Bowen ('22) on tetraploid spermatocytes in the pentatomid *Euschistus*, and more recently Fukushima ('31) on the corresponding phenomena in the pollen formation of *Brassica*.

² Paulmier ('99), Montgomery ('01, '06); also Wilson ('05, etc.), Gross ('06), Morrill ('10), McClung and Pinney ('12), and others.

⁴ Browne ('13, '15), Payne ('09, '10).

small size and long delayed synapsis; and, c) the sex chromosomes, which are in the female always of the XX type, in the male usually of the XO type.⁵

In all of the many species of this family that have been examined cytologically the three classes in question show a characteristic and constant grouping in the first spermatocyte metaphase, the autosomes being arranged in a well defined ring at the center of which is the small m-bivalent, while outside the ring, at a varying distance, lies the univalent X-chromosome (fig. 1, photos. 25, 28, 31). With only slight variations this arrangement is maintained with remarkable constancy, irrespective of variations in the number, size or other characteristics of the chromosomes. The same general pattern appears, for example in *Chariesterus* (diploid twenty-five), *Margus* (twenty-three), *Anasa* (twenty-one), *Archimerus* (fifteen), and *Alydus* or *Protenor* (thirteen). It is not affected by the size of the X-chromosome (small in *Alydus*, very large in *Protenor*) nor by the presence or absence of a Y-chromosome (photos. 28, 29). It is a singular fact that the ring pattern, as just outlined, appears only in the spermatocyte mitoses, and as a rule only in the first division (compare photos. 31, 32). In the gonial and somatic mitoses of neither sex is such a pattern shown, the metaphase chromosomes being scattered at random, and, strange to say, the same is true of the maturation divisions of the female (Morrill, '10). For these puzzling facts we have at present no explanation, and I have none to bring forward here.

In *Archimerus* the normal diploid number is fifteen, including twelve autosomes, two m-chromosomes and a single, unpaired X-chromosome. In the first normal spermatocyte division the autosome ring comprises six bivalents, with the very small m-bivalent at its center and the X-chromosome outside it, often at a considerable distance (fig. 1, photo. 25). In the tetraploid spermatocytes the number of each class of

⁵ A solitary exception is offered by *Metapodius* in which the male almost always is of the XY-type, through a few individuals have been found in which Y is missing (Wilson, '09 b).

chromosomes is doubled, but the same general pattern is followed, comprising a ring of twelve autosome bivalents, two m-bivalents at or near its center and two separate X-chromosomes outside it.⁶ In tabular form:

(a) Normal diploid Spermatogonia 15	Autosomes 12	m-chromosomes 2	X-chromosomes 1
(b) Normal first Spermatocytes 8	Autosome ring 6 bivalents	Center of ring 1 m-bivalent	Excentric 1 univalent
(c) Tetraploid Spermatocytes 16	Autosome ring 12 bivalents	Center of ring 2 m-bivalents (linked)	Excentric 2 univalents

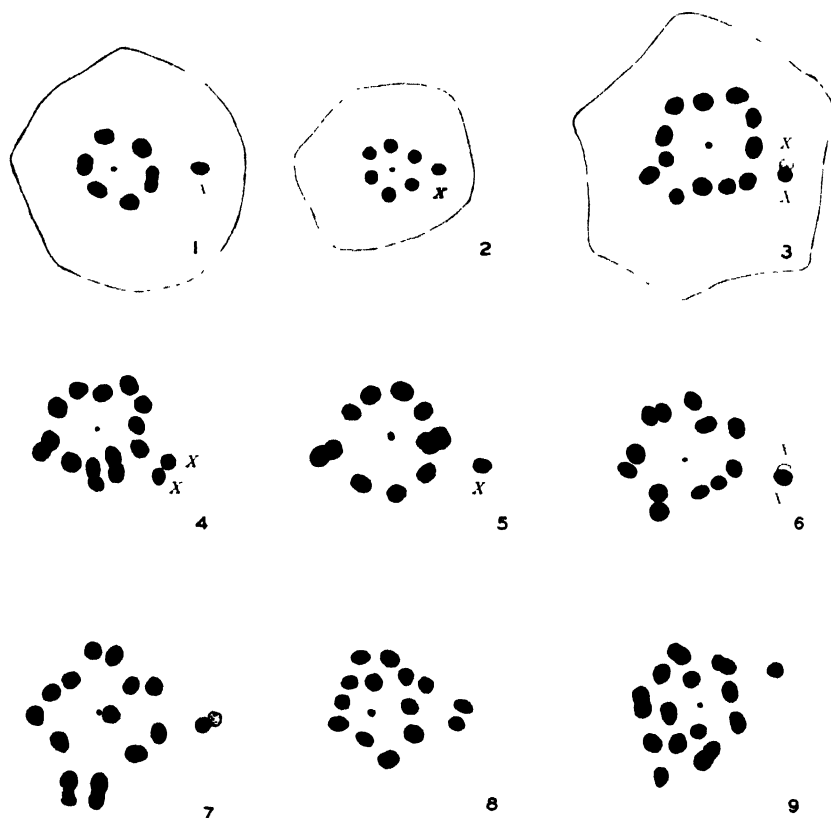
With some deviations the above grouping is evident in all the tetraploid metaphases of which a clear view can be obtained. Typical examples are shown in figures 3 to 9, which sufficiently illustrate the observed range of variation. In figures 3, 6, 8, and 9 the full double number appears (with exception of the m-chromosomes, as explained below). In four, one chromosome is missing; in five, two; while in seven, one extra one appears (perhaps due to a failure of synapsis). In nine the ring formation is hardly apparent; but such cases are rare, and none has been seen in which the ring pattern is completely obscured. When due allowance is made for slight variations in the time relations and for accidents of the technique the marked tendency to follow the normal ring pattern is a salient and unmistakable fact.

There are several interesting special features of the tetraploid metaphases, as follows:

1. A striking feature in all is the fact that the two m-bivalents are invariably linked end to end to form a linear quadrivalent chain lying exactly in the mitotic axis (figs. 11 to 14). Owing to this position the m-quadrivalent, when seen in polar view, does not differ in appearance from the m-bivalent in the normal cysts. When, therefore, the tetraploid metaphases are thus viewed the total number of

⁶In giving these numbers the so-called 'secondary' or 'equational' split is left out of account.

chromosomes in the group appears to be one less than twice the normal, i.e., fifteen instead of sixteen (figs. 3, 6). The true situation is only apparent in side views (figs. 11 to 14) when the quadrivalent condition of the m-complex is at once evident. No exception to this singular condition has been found. Unfortunately the number of late prophases is too



All original figures to the same scale, by camera lucida. Photographs (by the author) Zeiss 1-5 apochromat, Compens. 6, oc.

Figs. 1 to 14, Photos. 25 to 27, 33, 35, *Archimerus alternatus*, Say.

Fig. 1 Polar view, first normal spermatocyte metaphase.

Fig. 2 Polar view, normal second spermatocyte metaphase.

Fig. 3 Polar view, tetraploid first spermatocyte metaphase.

Figs. 4 to 9 Polar views, tetraploid spermatocyte metaphases, showing extreme range of variations in grouping.

small to show exactly how the m-chain is formed; but appearances indicate that the linkage, like synapsis in the normals, is delayed until the late prophase of the first division.⁷

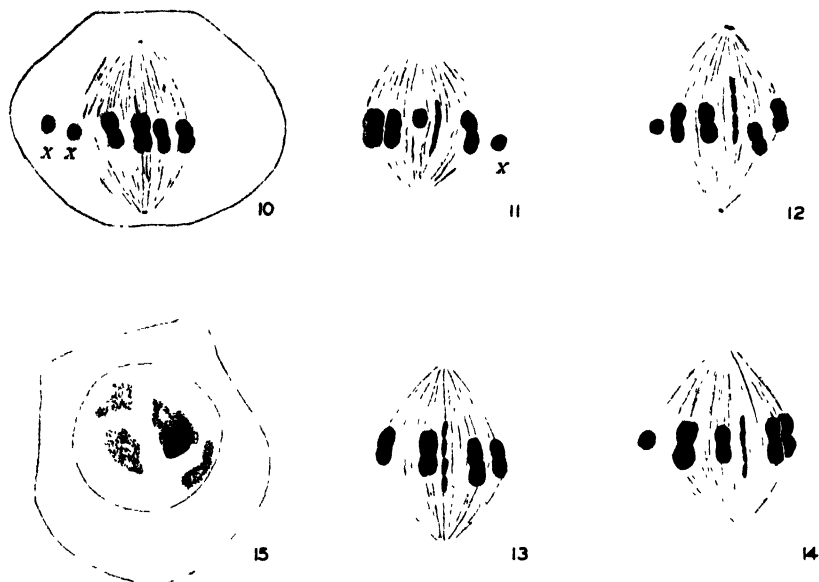
2. No less striking is the fact that the two X-chromosomes of the tetraploids are, so far as I can find, never united to form a bivalent, but remain separate, lying outside the autosome ring as in the normal spermatocytes.⁸ There is not the smallest doubt concerning the identification of these chromosomes, for, apart from their excentric position, they show in side view the same simple, rounded form (figs. 10 to 12, 14) as in the normals, with no trace of a bipartite or 'dumb-bell' shape. The surprising fact is thus made evident that although two homologous X-chromosomes are present in the male tetraploid groups (as in case of the normal diploid females) they must either have failed to conjugate in the synapsis or, if conjugation has occurred, it must subsequently have been undone by disjunction. It is, however, noteworthy that the two X's usually lie side by side and not far apart (figs. 3, 4, 6, 7, 8), sometimes nearly or quite superposed, so that the two can sometimes only be distinguished with certainty by careful focussing. In some cases only one X can be seen, probably because its mate lies outside the plane of section. On the whole, however, there is no doubt that two separate X's are most commonly, if not invariably, present, a fact which seems to indicate that somehow the synaptic process, or its sequel, is definitely affected by sex.

3. A third noteworthy fact is that in the earlier prophase of the tetraploid cyst, at a time when the autosomes are still rather diffuse and lightly staining, at least one compact and intensely staining chromosome nucleolus is undoubtedly present (fig. 15). This body is exactly similar to the pycnotic X-chromosome nucleolus in the neighboring normal cysts, and

⁷ See Gross ('04), Wilson ('09 a), etc.

⁸ The appearances shown in figures 3, 6, and 7 are due to the fact that the two X-chromosomes, though well separated, lie at different levels, so as to appear partially superposed in the drawings.

its identity cannot be doubted. Unluckily the material is too scanty for adequate study; and I have been unable even to make sure whether two such bodies are present or only one. In either case the facts are not favorable to the suggestion of Mohr ('15, '16) that pycnosis of the X-chromosome during the growth period of the spermatocytes is owing to the



Figs. 10 to 14 Side views of tetraploid first spermatocyte metaphases.

Fig. 10 Typical bivalents with two univalent X-chromosomes at one side. In 12 to 14, but one X appears. Typical m-quadrivalents in 11 to 14.

Fig. 15 Late auxocyte of tetraploid, showing one typical pycnotic X-chromosome (close beside it, in the same cyst, is a typical tetraploid metaphase).

absence of an homologous mate for this chromosome in the male sex.

4. As was to be expected, the tetraploid spermatocytes are, roughly speaking, about twice the size of the normal ones at the same stage (figs. 1, 3). This confirms the conclusion previously stated that the original doubling of number must have arisen in a single spermatogonial division and have been perpetuated thereafter by progressive normal bipolar divisions so as to produce an entire cyst of tetraploid descendants,

the normal karyokinetic ratio having been restored by a compensatory process of cytoplasmic growth.

COMMENT

The foregoing observations afford a striking demonstration of the individuality and genetic continuity of chromosomes; but their main interest lies in their bearing on the difficult and still obscure problem of chromosome movements and group formations. They do not, I fear, throw much light on those problems, but they may perhaps offer some suggestions for their further study. The fact that doubling the number of chromosomes does not destroy their typical plan of metaphase grouping demonstrates that this plan cannot be due to any preestablished structural relation between chromosomes and spindle, such as was long ago postulated by Rabl. In this respect the present results fall in line with numerous observations showing that the chromosome number may be varied experimentally without disturbance of the mitosis in other respects. On the other hand, the first impression given by the tetraploid metaphases is that their marked tendency to assume the normal grouping must involve their own active participation in producing the result, and that the moderate increase of deviations from the normal pattern which they show is a mechanical result of their increased number. I am in fact inclined to accept this interpretation, though with certain qualifications; and there is much in the recent literature of the subject that points in the same direction. Without attempting an extended review or hesitating to go over some old and familiar ground I will briefly consider some of the questions here involved.

In the earlier attempts to analyze the mechanism of cell division, the chromosomes were often treated, in effect, as if they were passively pulled (or pushed) about by contractile fibrillae ('Zugfasern,' 'traction fibers'), poleward currents, bipolar forces of attraction or the like, and a similar view was held or implied concerning the causation of the group formations of the chromosomes that appear in the late

prophases and metaphases. For example, the familiar simple ring patterns early observed in these stages of the urodele *Amphibia* by Flemming, Drüner, and their successors seemed to be a mechanical consequence of their assembly around the central spindle caused by the action of contractile fibrillae centering at the spindle poles. In later years it was often assumed that the arrangement of the chromosomes at metaphase might, in like manner be due to simple mechanical factors, such as the crowding of the chromosomes. For example, it is a familiar fact that when the chromosomes are of different sizes the smaller ones usually tend to mass toward the center of the group with the larger ones surrounding them.⁹ This condition is even now commonly treated as a simple mechanical effect;¹⁰ but, as will presently be shown, the inadequacy of this long ago became evident. R. S. Lillie, in his well-known work on this subject ('05), approached the problem from the standpoint of mutual attractions and repulsions in colloid aggregates, employing as physical models the earlier experiments of Mayer on floating magnetized needles. If a group of such needles, separate and free to move, be floated upright with like poles adjoining, the units tend, of course, to move apart because of their mutual repulsion. If, however, they be drawn together by the attraction of a larger overhead magnetic pole of opposite sign they fall into patterns some of which are practically identical with some of the simpler ones assumed by the metaphase chromosomes of various species. This comparison has been extended by several later writers. Cannon ('23) emphasized the close similarity of pattern in the two cases as modified by corresponding variations in the number of units, and similar results have been reached by Kuwada ('29) and Levan ('29). The results of this work leave hardly a doubt that comparatively

⁹ See, for example, figures 394 to 396, 413, etc., in my general work 'The Cell.' 3rd ed., '25-'28.

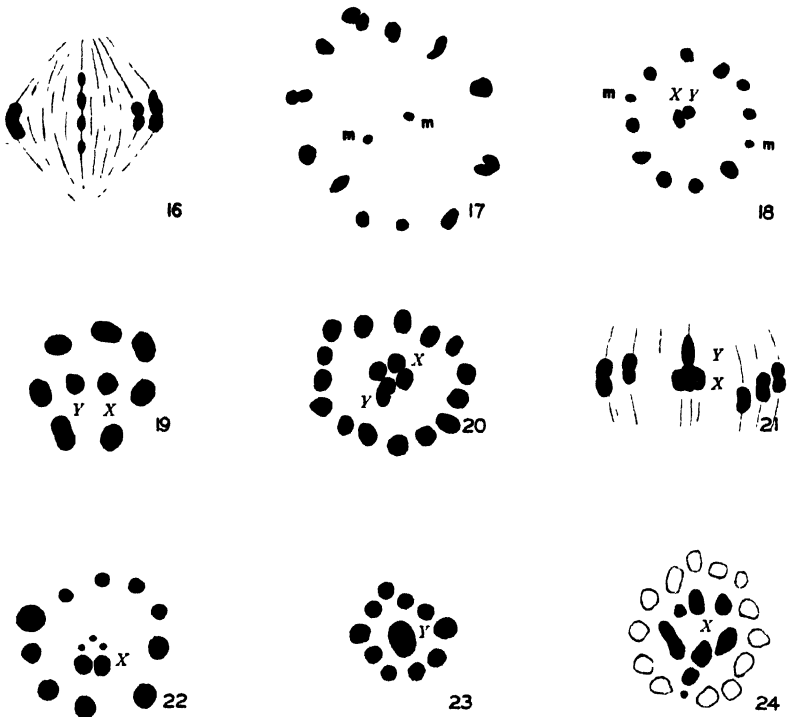
¹⁰ Compare Tischler, '22, page 328: "Wenn wir Chromosomen von verschiedener Grösse vor uns haben pflegen sich die grösseren nach aussen, die kleineren nach innen in der Platte einzustellen. Die Erscheinung ist offenbar durch rein mechanische Momente bedingt."

simple physical or mechanical factors external to the chromosomes do in fact play an important part in the movements and grouping of the latter. On the other hand, if the operation of these models has any real bearing on the phenomena of mitosis it indicates no less clearly that the rôle of the chromosomes in the process is not a merely passive one, for the operation of the model is of course absolutely dependent on the fact that the units repel one another. Lillie assumed such a mutual repulsion on the part of the chromosomes owing to their like electrical charge, and a corresponding assumption has been made, at least tentatively, in some of the most recent work on the subject.¹¹ A wider survey of the facts leaves, however, no room for doubt that beyond these simple factors other and less obvious ones are involved.

An example of this is offered by the accurate serial alignment of the m-chromosomes to form the characteristic axial chain in the tetraploid spermatocytes of *Archimerus*, an arrangement that finds no analogue in the magnetic or other physical models. This condition, though rather uncommon, is by no means without a parallel. In the sixth of my 'Studies on Chromosomes,' for example ('10), I described a single individual of the coreid *Metapodius femoratus* in which every mitosis shows the presence of three m-chromosomes instead of the normal two. This is plainly seen in both the spermatogonial and the spermatocyte divisions, but with an important difference. In the former case the three are scattered at random among the autosomes, but in the first spermatocytes at full metaphase they are always linked end to end to form an axial chain of three lying exactly at the center of the autosome ring (photo. 34). This condition is exactly analogous to that presented by the axial tetraploid chain in *Archimerus*. Equally close is the analogy offered by the conditions seen in the normal first spermatocyte divisions of *Notonecta*, a genus of Heteroptera made known cytologically by the interesting comparative studies of Browne ('13, '15). Of six species examined, five show in the first division a fine autosome ring

¹¹ Compare Bleier, '30, '31.

with either one or two small bivalents lying inside it (fig. 17). In species *N. undulata* and *indica*, these two commonly lie side by side and in this position divide separately; but frequently they are linked end to end to form a linear axial chain of four components essentially similar to that invariably displayed in the tetraploids of *Archimerus* (fig. 16).



Figs. 16 to 24 Normal spermatocyte metaphase groups from various sources.

Fig. 16 Side view, first division, *Notonecta undulata* (Browne).

Fig. 17 Polar view, first metaphase, *Notonecta shooteri* (Browne).

Fig. 18 Second metaphase of same species (Browne).

Fig. 19 First metaphase of lygacid *Oncopeltus* (Wilson).

Fig. 20 Second metaphase, *Gelastocoris oculatus* (Payne).

Fig. 21 The same, side view (Payne).

Fig. 22 Second early anaphase of *Acholla multispinosa* X-group (Payne).

Fig. 23 The same, Y-group (Payne).

Fig. 24 *Contracoecum* (*Ascaris*). Polar view, second metaphase. 8-fold X-group within the ring (Goodrich).

The foregoing cases of linear alignment are so similar that we can hardly doubt their similar causation. Conditions more complicated, but perhaps similarly caused, are displayed in *Protortonia*, a coccid hemipter recently studied with care by F. Schrader ('31). In this astonishing case the diploid chromosome number is five in the male and six in the female. In the second spermatocyte metaphases all of the five univalent chromosomes are accurately aligned to form an axial chain lying exactly in the mitotic axis. In the ensuing stages the chain divides transversely in such a manner that two components pass to one pole and three to the other (photos. 36a, 36 b). The study of numerous cases leaves no room for doubt concerning the regularity and normality of this unique process.

In none of the foregoing cases, least of all in the last one, have we any explanation of the singular configuration in question, and certainly none of them is in conformity with existing attempts to explain metaphase patterns. It may be urged that these are exceptional cases, and the fact is indisputable; but nothing is more certain than that somehow they must fit with groupings of more conventional type. We might imagine that such types of behavior are caused by special conditions in the spindle, for instance by poleward strains or currents such as have sometimes been held responsible for the more usual forms of anaphase movements, whether on the part of daughter chromosomes or of nucleolar fragments. For all we know such may in fact be the explanation; but in considering such a possibility we must reckon with the well-known and no less striking end-to-end serial alignments that often occur in the prophases both of mitosis and of meiosis prior to the formation of a spindle. This fact has long been familiar in higher plants (*Carex*, *Oenothera*, etc.) and even in certain higher animals (e.g., in the prophases of spermatogenesis in the toad (King, '07) and was formerly regarded as a consequence of telosynapsis. In such cases we can hardly escape the conclusion that the chromosomes are themselves responsible, in some degree and manner, for the grouping which they assume.

When a spindle is present, even in its incipient stages, the case is more doubtful; but even here close study brings out many facts that point to a similar conclusion. A simple case in point is offered by the characteristically eccentric position of the X-chromosome in the first spermatocyte division of the coreids. Nothing has been found in the visible characteristics of this chromosome to explain its behavior in this respect. It is independent of the order of division—in most species X divides only in the first division, passing undivided to one pole in the second (*Anasa*, *Syromastes*), but in a few forms for no apparent reason, the reverse order is followed (*Archimerus*, *Pachylis*). The same position is taken whether a Y-chromosome be present (*Metapodius*) or absent (all other known cases). It is independent of size, shape, or other visible characteristics. And since it is always this particular chromosome that assumes the eccentric position its characteristic performance must somehow be conditioned by its specific nature.

The case is similar in respect to the small m-bivalent in the coreids. It might, for instance, be assumed that the invariably central position of this chromosome is a mechanical result of its small size—that this is only a particular case of the above-mentioned tendency of small chromosomes to collect in the center of the group. That such groupings may often be affected by simple mechanical conditions seems hardly open to doubt; but the rule meets with too many contradictions¹² to have much explanatory value. For example the m-chromosomes, as earlier indicated, occupy a wholly inconstant position in the spermatogonial divisions, commonly also in the second spermatocyte divisions (photo. 32) and in all the divisions of the female cells.

Still more impressive are the facts shown in the ring patterns of other families of Hemiptera, in particular the *Notonectidae*, *Gelastocoridae*, and *Reduviidae*, some of them

¹² See, for instance, Harrison and Doncaster ('14) on the chromosomes of hybrid moths.

among the most remarkable known.¹³ Though these cases have long been known, it will be useful to emphasize certain of the facts here. In *Notonecta*, as shown by Browne, most of the species examined show a fine, open ring pattern in both spermatocyte divisions. In the first division of *N. insulata* and *N. shooteri* two small bivalents, resembling the coreid m-chromosomes, lie inside the ring (fig. 17), while the X- and Y-chromosomes (at this time unrecognizable) form part of the ring itself. In the ensuing second division all this is changed. Although the ring is still perfectly formed, a re-grouping has taken place such that the two small chromosomes (readily distinguishable by their size) now lie in the ring itself, while their place at the center has been taken by the unequal XY-pair (fig. 18).

In this case too, it is plain to demonstration that it is not merely the size or shape of the chromosomes that determine their position, but a less obvious reaction in which their specific quality is involved. The same situation is even more strikingly displayed in certain Reduviidae and Gelastocoridae which, as shown by the remarkable observations of Payne ('09, '10), offer some magnificent examples of ring patterns, though the details are different from the foregoing. In most of these cases the ring formation first appears in the second division, where it is displayed with almost schematic clearness with the unequal XY-pair always at its center. This grouping, again, is independent of the visible composition of the XY-pair. In all cases Y is a single chromosome, which varies only in respect to size and shape, whereas the X-chromosome displays an astonishing diversity. In *Diplocodus* both X and Y are single chromosomes, slightly unequal, much as in the *Pentatomidae* or *Lygaeidae* (compare fig. 19). In *Fitchia* X is composed of two components, in *Prionidus* of three, in *Sinea* and *Gelastocoris* of four (figs. 20, 21) and in *Acholla multispinosa* of five (fig. 22). In the latter remarkable case Y, since as always, is the largest of all the chromo-

¹³ It should be borne in mind that in all these groups the ring patterns differ markedly in detail from those of the coreids.

somes (fig. 23), while X characteristically consists of five components, two large and three very small. A climax appears in *Contracoecum* (*Ascaris*) *incurva*, a nematode (fig. 24) where a single, small Y is opposed by an X-group of no less than eight components showing constant size differences, but acting as a unit (Goodrich, '16). Despite all this diversity the XY-pair always lies at the center of a sharply marked autosome ring, the space within which in *Contracoecum* is almost completely filled by the huge XY complex (black in fig. 24). All this makes it clear to demonstrate that the position of particular chromosome pairs or complexes in the metaphase group is not determined as a mere matter of size, shape, or number, but involves a selective activity, more or less accurate, in which the chromosomes must themselves be involved.

Such facts, which I think have not yet received the attention they deserve, should help to broaden our conceptions concerning the mechanism of mitosis. The cytologists of the nineties, following the lead of Van Beneden, Bütschli, Boveri, and other early masters, often found it convenient to treat mitosis as a dual process involving two distinct series of events, namely, on the one hand the activities of the chromosomes and on the other those of the 'achromatic figure' (spindle, amphiaster, central bodies), which normally run closely parallel, but are in considerable measure experimentally separable. As part of this conception it was commonly assumed that the achromatic figure is, so to speak, the prime mover in mitosis, being in particular responsible for the movements and groupings of the chromosomes, processes in which the chromosomes, by implication at least, were treated as passive bodies. The usefulness of this procedure in practice has been proved by a long series of fruitful researches. It was, however, only a first attack on an intricate problem of cell mechanics, developed at a time when, except for the early splitting of the spireme threads, very little was known about the complicated activities of which the chromosomes are capable prior to the formation of a spindle.

Such, for instance, are their amazing maneuvers during the presynaptic and synaptic periods—their accurate side by side conjugation in synapsis and their closely related pairing in the somatic mitoses of the *Diptera*; their serial alignments and linkages; their precisely ordered exchanges of material in the operations of crossing-over. Prominent among these activities are the spectacular performances of the chromosomes in diakinesis and metakinesis, such as those which astonish us in the serial alignments and ring linkages in *Oenothera*, varying in a definite manner from species to species. To ask us to believe that their activities suddenly cease with the breakdown of the nuclear membrane and the operations of metakinesis places too great a strain on our credulity.

In case of hemipteran spermatocytes, obviously, we cannot make use of Boveri's principle of the persistence of chromosome grouping from telophase through the interphase up to the ensuing prophase;¹⁴ for, as has been indicated, the pattern changes completely between the last spermatogonial and the first spermatocyte divisions and commonly also between the first and second of the latter (compare photos. 31, 32). The interphase or prophase must therefore be in these cases the arena of active shiftings of the chromosomes, as in so strikingly demonstrated by the spermatocyte divisions, for instance, of *Notonecta*, *Gelastocoris*, *Acholla*, or *Contracoecum*.

Great uncertainty, however, still prevails in respect to the causes of these movements, both in mitosis and meiosis. A considerable number of recent observers have maintained that they are autonomous on the part of the chromosomes throughout the whole mitotic cycle, for example, Robyns.¹⁵

¹⁴ This principle, brilliantly demonstrated by Boveri in the early cleavage of *Ascaris*, has been shown to hold true in some of the higher plants by Kagawa ('26) and by Körperich ('30).

¹⁵ "C'est par un mouvement autonome que les chromosomes tendent à se grouper dans le plan équatorial . . . Il nous paraît que la disassociation dikinétiq̃ue des chromosomes est due, elle aussi, de même que l'arrangement équatorial des chromosomes mères, à un mouvement autonome des chromosomes" (Robyns, '29, pp. 173-174).

The view of Bleier is similar in principle, both the prophase movements and those of the metaphase and anaphase being ascribed to autonomous forces of mutual repulsion, as in the conception of Lillie, though with the prudent reservation that other forces may also be operative.¹⁶ Both authors ascribe only a sustaining and orienting function to the spindle and deny the existence of traction fibers. On the other hand, there is no lack of observers who still hold that, apart from the initial separation of the daughter chromosomes at the beginning of the anaphase, the active agent in these activities is the spindle (whether fibrillar or not). Wassermann ('26) ascribes the anaphasic movements to poleward currents in the spindle caused by changes of viscosity in its substance; Schaefer ('25, '27) to vortical currents in the spindle substance; Bělař ('27, '29) to a primary activity of traction fibers attached to the spindle, followed by an active elongation of the spindle, which thus acts as a 'Stemmkörper' or pushing agent by which the poles (and with them the daughter chromosomes) are forced farther and farther apart.

Such differences of recent opinion (not to cite others) emphasize the uncertainties that still hang over even the most obvious of the questions here at issue. I will, however, review briefly certain other facts and hypotheses that are of importance for further inquiry. The most interesting of these are concerned with the possible genetic relations between chromosomes and spindle substance. The earlier studies on mitosis, in particular those dealing with the central spindle type in urodeles, seemed to show that the spindle is originally formed independently of the chromosomes, though in the end it becomes closely correlated with them. Almost from the beginning, however, other possibilities were suggested by the phenomena of intranuclear spindle formation, a process first demonstrated by Van Beneden and by Boveri in the polar

¹⁶ "Nun treten zwischen den Längshälften Abstosskräfte auf, die Chromosomen in der Aequator als einer Gleichgewichtszone führen, wobei vielleicht andere Kräfte mitwirken. . . . Die Abstosskräfte führen nun zur vollständigen Trennung der Längshälfte und deren Wanderung an die Pole" (Bleier, '30, p. 134).

spindles of *Ascaris* and long afterward found to be characteristic of the corresponding mitoses in arthropods and chordates generally, as well as in both the meiotic and somatic mitosis of the higher plants. Recent studies in this direction have produced specific evidence which demonstrates so close an original association between spindle substance and chromosomes as to suggest a common origin for both. This subject may most readily be approached from the interesting hypothesis of the late lamented Bělař ('27, '29). It was his novel assumption that each traction fiber (Zugfaser) is directly derived from a corresponding chromosome, being as it were spun out from the latter at its point of attachment to the spindle. A plausible solution is thus offered of the long-standing puzzle offered by the precise adjustment between each spindle 'fiber' and its corresponding point of attachment, the latter being nearly or quite constant for each chromosome and often marked in advance by visible structural formations (polar granules, constrictions) clearly evident long prior to the breakdown of the nucleus and the beginning of spindle formation. Bělař thus made the chromosomes themselves responsible for an important part of their own movements, including both their metaphase arrangements and the earlier poleward movements of the anaphase.¹⁷

Unfortunately we cannot make much use of this ingenious hypothesis in its original form; it was indeed hardly more than a theoretical construction *ad hoc*, unsupported by adequate evidence. But even though it may not be acceptable in all its detail it is of much interest in connection with other recent attempts to bring the formation and functions of the spindle into more direct organic connection with the chromosomes. Apart from the facts in anastral mitosis there is evidence that even in amphiastral mitosis the definitive spindle, or an important part of it, may be of intranuclear origin. It has long been known that in this type of mitosis

¹⁷ Die Einordnung der Chromosomen in dem Spindelaequator und das Auseinanderweichen der Tochterchromosomen beruhen nach dieser Hypothese auf Zugwirkungen der Zugfasern, das Auseinanderweichen der Tochterchromosomen beruht ausserdem noch auf Stemmwirkung des Stemmkörpers'' ('29, p. 461).

the original spindle often disappears, to be replaced by a new one formed between the persistent centers lying on or near the nuclear membrane and more or less widely separated. Familiar examples of this are offered by the history of the sperm asters and their relation to the first cleavage figure in many animals, both vertebrate and invertebrate. I myself concluded nearly forty years ago in such a case (sea-urchin) that the fibers of the first cleavage spindle, which succeeds the sperm amphiaster, "do not really grow into the nucleus from without, but are differentiated in situ out of the acromatic network."¹⁸ Equally striking are the well known similar conditions shown in the early cleavage blastomeres in many animals, where in the late anaphases and telophases a small daughter amphiaster is formed within the mother amphiaster at each pole, but later disappears, being replaced by a new amphiaster formed between the persistent centers lying at or near the nuclear poles. In such cases, too, there is, I think, much force in Wassermann's contention that the new spindle is, at least in large part, of intranuclear origin. As this author has indicated, strong special evidence for this conclusion is offered by the conditions in gonomery; for, as shown by Rückert's early work on the copepod egg ('95), when the gamete nuclei fail to fuse before the first cleavage, two separate spindles may be formed, lying side by side, though only one center is present at each pole. A nearly similar condition is shown by Conklin ('01) in the first cleavage of *Crepidula*. Here, too, may be mentioned the fact, observed by Juel and others in plant mitoses, that a single chromosome, accidentally separated from its fellows, may produce a diminutive but functional spindle.¹⁹ Somewhat similar conditions appear also in the polar mitoses of the eggs of *Cyclops* where, as shown by Schiller ('95), after slight etherization (and sometimes even in the untreated eggs), the spindles appear compound as if composed of several smaller spindles lying side by side, each corresponding to one or a

¹⁸ Wilson, '95, p. 453.

¹⁹ Compare Wilson, 'The Cell,' '25, figure 391.

few chromosomes. In all such cases it would seem to follow that the spindle substance must be traceable to the chromosomes from which the nucleus is originally built up. If this be granted, Bělař's assumption concerning the origin of the 'traction fibers' is anomalous only in the assumption that they are given off from the chromosomes in so direct a manner and at so late a period. His hypothesis is, however, only one of many recent attempts to elucidate the origin of the spindle substance and its possible genetic connection with the chromosomes.²⁰

The question here turns largely on the origin of the 'achromatic' basis or ground substance of the interphase nucleus from which, in the belief of many cytologists, the spindle substance is derived. Current opinions on this question fall into two groups. One of these includes the view that the ground substance is derived, wholly or in part, from the chromosomes themselves. Here are comprised most of the studies on the chromonema theory, ranging from the well-known earlier papers of Bonnevie ('08, '13) and of Vejdovsky ('11-'12) down to the more recent ones of Martens ('25, '27, '29), Kauffmann ('26, '29 b), Sharp ('29), Hedayetullah ('31), and others. These observers have made us familiar with the fact that the chromosomes show a dual structure, consisting of a strongly chromatic thread, single or double (chromonema or dolichonema) surrounded by a more lightly staining envelope or 'matrix'; further, that in the telophases the substance of the matrix gives rise or contributes to the 'achromatic' substance of the interphase nucleus, though the latter may also receive accessions from other sources. Here,

²⁰ I do not here speak of spindle fibers since their existence as preformed structures in the living cell, never satisfactorily demonstrated, is at present denied by many observers. Compare Robyns ('24, '29), Martens ('29, '30), Bleier ('29, '30), Jungers ('31), and others. No one doubts, I suppose, that the regular fibrillar structure of the coagulated spindle is conditioned by a corresponding bipolar orientation of some sort in the living object.

obviously, is given a possibility that the spindle substance may be derived, indirectly at least, from the chromosomes.²¹

A second group includes Körperich ('30) and Bleier ('30, '31), who adopt a far-reaching hypothesis of nuclear dualism. Both authors assume that the spindle substance has no genetic connection with the chromosomes, but only accompanies them closely at every stage in the mitotic cycle. This material, lightly staining, is called by Körperich 'substance parachromosomatique,' and by Bleier 'paragenoplastin.' Assumed by both authors to surround and permeate the chromosomes of every stage, this substance is supposed to play an essential part in their maintenance and activities, being necessary for their poleward orientation and the full accomplishment of their normal movements. As earlier indicated, Bleier, who has most fully elaborated the hypothesis, considers the movements of the chromosomes to be autonomous, the spindle being responsible only for their bipolar orientation. Gates, in his latest paper ('32), holds 'the real unit in mitosis is the chromosome together with its attached spindle fibre, whatever that may be.' This conception is obviously closely akin to that of Bělař, though the possible genetic relation between spindle fiber and chromosome is not considered. Gates adds the suggestion that "the configurations of the mitotic figure derive their unity from the fact that the various chromosomes go through their evolutions simultaneously but independently" (op. cit., p. 6). This statement seems to me to be on the whole in harmony with the facts discussed in the foregoing pages. Perhaps it may, in particular, offer a clue to the puzzling fact that the tetraploid metaphase patterns

²¹ Martens holds that the chromosomes retain their identity throughout the interphase nucleus, its achromatic basis being identical with the matrix of the telophase chromosomes. The spindle-forming substance is, however, considered by him to be a new formation between the chromosomes, though he admits the possibility that this substance may receive accessions from the chromosomes. "Il est varisemble que, dès le début de la telophase, la substance fusorial devienne miscible avec certains éléments au moins des chromosomes (probablement leur substratum achromatique." He conjectures, however, that at the beginning of the following prophase the two substances may separate ('30, p. 206).

here made known tend so strongly to follow those displayed in the corresponding normal diploid mitoses.²²

As will appear from the foregoing brief survey, current opinion on this subject is still in a very unsettled state. I think, nevertheless, that most of the facts point to a much closer association between chromosomes and spindle substance throughout the mitotic cycle than was formerly supposed, and that we must reckon seriously with the possibility that that substance may be directly derived from the chromosomes, as Bělař assumed in case of the traction fibers. Important evidence pointing in this direction has been brought forward of late by the studies of Hughes-Schrader and F. Schrader on the maturation divisions of certain insects. Foremost in interest among these, perhaps, is the work of the first named observer ('24) on the first polar spindle in the egg of the strepsipteran *Acroschismus*, whose preparations I have had opportunity to examine. In a rather late prophase, at a time when the bivalent chromosomes are quite irregularly scattered, each of them lies at the center of a separate small spindle, the group showing no trace of common orientation or polarized arrangement. Only later do they draw together, become parallel, and unite side by side to form a single composite spindle. Here the close original association with each chromosome (if it be not the actual origin from it) of its quota of spindle substance is an obvious fact—one on which Hughes-Schrader has laid especial emphasis, with full recognition of its significance.²³ Second

²² It is proper to add that Gates' paper was first received after the present one was in manuscript, nearly ready for the press and substantially in its present form; also that a paper developing conclusions in the same general direction had already been sent to press by my colleague Prof F. Schrader.

²³ This author recalls the perhaps similar condition earlier observed by Smallwood ('04) in the amphiastral polar spindles of the nudibranch *Haminia*. To this may be added the strange fact, described by King ('08) in the first polar mitosis of the toad's egg, that in a rather late prophase, prior to the breakdown of the nuclear membrane, each chromosome is accompanied by a very definite small aster, a fact especially noteworthy because this mitosis, as in chordates generally, is of the anastral type. Perhaps the curious phenomena of 'merokinesis,' described by Reuter ('09) may be of related type.

in importance only to this case are those of the coccid Hemiptera *Llaveia* (Hughes-Schrader, '31) and *Protortonia* (F. Schrader, '31) in both of which there is almost equally strong ground for the conclusion that each spindle 'fiber' may be directly derived from a corresponding chromosome. If these and the preceding observations do not conclusively demonstrate this conclusion they do establish the fact that in respect to their origin at least, spindle substance and chromosomes are so closely associated as to constitute an integral organic unit.²⁴

In emphasizing the active participation of the chromosomes in their own movements and groupings I have not wished to imply their absolute autonomy. On the contrary, I find it impossible to doubt that the spindle substance also plays an important rôle in the phenomena; and further (assuming with Körperich and Bleier that this substance may persist in the interphase nucleus) it seems quite possible that it may likewise be involved in those activities of the chromosomes that take place in the absence of a formed spindle, e.g., in the performances of the synaptic or the prophasic periods. If, however, the foregoing considerations have any weight—that is to say, if at certain stages the spindle substance actually forms a component part of the chromosomes—the question of 'autonomy' on the part of the chromosomes in contradistinction to the spindle substance becomes largely one of words. In point of fact few if any writers, I think, have attempted to restrict the term 'chromosome' to the chromonema or chromomeres, but have included with these the more lightly staining 'matrix' in which they lie. The questions here involved have obviously a broader interest in their possible bearing on the long debated subject of the participation of the nucleus (i.e., the chromosomes) in the general constructive activities of the cell.²⁵ As regards the more special problem of chromosome movements and groupings the

²⁴ Compare Gates (op.cit.).

²⁵ Compare 'The Cell,' '25, pp. 65 ff and (in greater detail) Alexander and Bridges ('26), pp. 39, 53.

evident fact remains that we still have no adequate understanding of the phenomena. What has here been offered is no more than a point of view from which we may possibly make closer approach to the study of these interesting, but still mysterious, phenomena. From the standpoint of precise physicochemical investigation the history and relations of chromosomes and spindle substance is still full of uncertainties which call for more adequate analysis than they have yet received.

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PLATE

PLATE 1

EXPLANATION OF FIGURES

25 to 36 Photomicrographs by the author of spermatocyte metaphase patterns of various types. All taken with Zeiss apochromatic 1.5, oil immersion and compens. 6, oc.

25 *Archimerus alternatus*, normal first metaphase (compare fig. 1).

26 The same, normal second metaphase, exceptionally showing same grouping as in first division (compare fig. 2, contrast fig. 32).

27 Corresponding view, first tetraploid metaphase; only one X-chromosome seen (at right), the other being at lower level (compare fig. 3).

28 Normal first metaphase of *Metapodius terminalis* (no Y-chromosome) (from slide by Montgomery, see Wilson, '09 b).

29 Corresponding view of individual with both X and Y (Wilson, '09 b).

30 Second metaphase of *Gelastocoris*, with quadripartite X-group (published in Payne, '08).

31 First metaphase, *Syromastes* (from Wilson, '09 a), X double.

32 Second metaphase of same, typical grouping (Wilson ('09 a).

33 Normal first metaphase, *Archimerus*, side view, showing two autosome-bivalents, m-bivalent, and univalent X-chromosome.

34 Similar view, first metaphase of *Metapodius femoratus* with m-trivalent (compare Wilson, '10).

35 Similar view of *Archimerus* tetraploid, showing two autosome bivalents and central m-quadrivalent (compare figs. 11 to 14).

36 a Second metaphase of normal *Protortonia* in side view, showing axial chain of five chromosomes (from a preparation by F. Schrader).

36 The same slightly later, showing division of the pentad chain (same source).



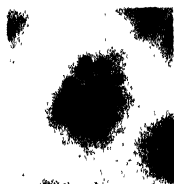
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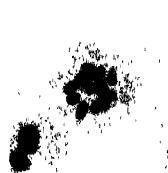
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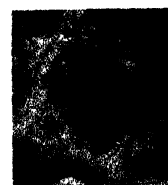
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OBSERVATIONS ON THE LIFE HISTORY OF A FINGERNAIL SHELL OF THE GENUS SPHAERIUM¹

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THREE PLATES (EIGHTEEN FIGURES)

AUTHOR'S ABSTRACT

By statistical analysis and microscopical examination of a series of monthly quantitative collections, an attempt has been made to interpret the life history and reproductive habits of *Sphaerium solidum*, one of the numerous species of the genus *Sphaerium*.

Individuals of the species under observation have a distinctly limited life span of approximately one year. The period of maximum reproduction of this species does not occur in the summer months, as previously believed, but in the winter months. Seasonal growth rings are not present on this shell, although concentric lines are characteristic. No individuals of maximum size were present during the months from August to February. Maximum-size adults are apparently sterile. The analysis of distribution curves of each quantitative sample indicates two size groups in each collection as shown by the bimodal character of each graph. Distribution curves of embryos feature the bimodal character of the young and adult. The conclusions are founded on data secured from twelve monthly collections made from the same habitat totaling 7022 individuals.

The fingernail shells, or Sphaeriidae, have long been known to undergo direct development, but detailed life-history studies have never been published for any American representative of this family. It is common knowledge that the genera *Sphaerium*, *Musculium*, and *Pisidium* of the Sphaeriidae are represented by numerous species, all of which are hermaphroditic and viviparous, but specific life-history information is lacking.

In 1896, Gilman A. Drew contributed a short description on the anatomy of *Sphaerium sulcatum*. Later, Ralph J. Gilmore ('17) published a paper on reproduction and growth of two species of the Sphaeriidae. A more extensive study of a member of this family has recently been carried on by Cecil R. Monk ('28), whose paper contains the results of an anatomical and preliminary life-history investigation of

¹ Contribution from the Zoölogical Laboratory of the University of Illinois, no. 429.

one species in this same family. Except for incidental reference to isolated observations, these three papers comprise the only knowledge available in English on the anatomy and life history of this molluscan family.

Several European workers have been concerned with the embryological and anatomical features of related European species. As early as 1828 Jacobsen recorded the fact that young develop in pouches, although in error as to the relation of the young to the marsupium. Oskar Schmidt ('54) investigated the anatomy of *Cyclas calyculata*. In this and in succeeding references to early investigations, reference is made to the genus *Cyclas* because this generic name appears in much of the older literature on the genus which now goes under the valid name of *Sphaerium*. Dr. Franz Leidig made the first general anatomical study in 1855 when he reported the information derived from a study of the young and more or less transparent specimens of *Cyclas cornea*. Stepanoff ('65) was the first to point out the brood pouch of *Cyclas* in its true relation and his results received the confirmation of Ziegler ('85). The work of both these investigators was directed to the study of the segmentation and development of the embryo, the notes on the brood pouch being of a casual nature. De Bruyne ('98) figures a brood pouch in a study of phagocytosis. Poyarkoff ('10) and Schereschewsky ('11) were concerned with the cellular structure of the brood pouch, its origin and function, when dealing with the formation of the embryos of *Cyclas*. The latest and perhaps most important European papers are those of Max Egon Thiel, a German investigator, who has made a comprehensive study ('26) of the growth, reproduction, and life history of *Sphaerium corneum*, directing his observations chiefly to seasonal growth.

All of the above-mentioned papers are concerned primarily with anatomical or embryological investigations of the Sphaeriidae. In all the literature there is a lack of specific material concerning the life history and reproductive habits. Recent publications on the Mollusca are replete with refer-

ences to the scanty information available on the life cycle of the Sphaeriidae. Believing that valuable information may be derived from a statistical study of a single species, Dr. H. J. Van Cleave, of the University of Illinois, suggested this study.

The writer is indebted to Dr. H. J. Van Cleave, under whose direction the study was conducted, for his assistance and guidance throughout the progress of the work. I also wish to thank Dr. V. Sterki for the identification of the specimens, and others who have given assistance in collecting in the field. Mr. Frank C. Baker has been especially helpful by offering suggestions in the interpretation of results secured.

MATERIAL

In preliminary field studies Sphaeriidae were found in relative abundance in a number of streams in the vicinity of Urbana, Illinois. Most of these localities had been under observation for a number of years, and many collections submitted to Dr. V. Sterki for identification had revealed the fact that most of the habitats carried mixed populations of two or more species of the genus. Because of the difficulty in separating some of the closely related species, mixed populations furnish serious obstacles to a specific study of the life history. Consequently, it became highly desirable to locate a place where a single species only could be found. An ox-bow pond near Muncie, Illinois, met the requirement, and a series of monthly quantitative collections were taken, beginning in February of 1930 and extending to March of 1931. Specimens of this material submitted to Dr. V. Sterki were identified by him as *Sphaerium solidulum* (Prime).

METHODS

An attempt was made to collect at regular monthly intervals. To have the collections representative, selected new areas from the same general habitat were always chosen.

The equivalent of from four to fifteen square feet of bottom was dredged each month. The sphaeriids were brought into the laboratory, where they were killed by immersing in boiling water and were then preserved in 70 per cent alcohol.

A vernier slide micrometer was used in measuring the shells. Microscopical measurements were taken with the aid of an ocular micrometer.

Observations and measurements were made on a total of 7022 individuals in the course of the year's investigation, and the results are presented in figures 1 to 12. In order to give validity to the study, no sample of less than 350 individuals was studied, and, in most instances, each contained at least 500 specimens.

A preliminary study revealed the fact that increments of 0.5 mm. represented the size differences best suited to a statistical analysis of population and individual growth rate in this species. These groups were arrived at in this study by selecting fifty individuals of the first collection ranging in size from the largest to the smallest and taking the measurements of their length, width, and thickness. Table 1 shows the results of this study.

It was found that the greatest degree of accuracy prevailed for measurements of thickness because of ease in measuring and also because the edge of the shell tends to crumble in the jaws of the vernier caliper when length and depth of the shell are being determined. This was especially noticeable in handling young shells. From inspection of table 1, it is noticed that on the basis of thickness, specimens can be divided into eighteen different groups, ranging in this dimension from 1.5 mm. to 10 mm. Accordingly, these eighteen size groups were established for the remainder of the study. Plate 1 (near actual size) illustrates the size groups. The shells from which this plate was made were selected from a single collection and arranged in an ascending scale of size. The last specimen, in the lower right-hand corner of plate 1, is an individual selected from the 8.0-mm. group to illustrate the relation of length to thickness.

Distribution curves of each collection were prepared, plotting numbers against size groups after each collection was reduced to the common basis of 1000 individuals. On this scale direct comparison may be made.

TABLE 1

Showing the relation of the three dimensions of fifty individuals of S. solidulum expressed in millimeters

INDIVIDUAL NO.	THICKNESS, MM.	WIDTH, IN MM.	LENGTH, IN MM.	INDIVIDUAL NO.	THICKNESS, MM.	WIDTH, IN MM.	LENGTH, IN MM.
1	2.0	3.2	3.9	26	6.7	8.0	10.2
2	2.3	3.7	4.3	27	6.8	8.2	10.3
3	2.5	3.9	4.6	28	7.0	8.4	10.6
4	2.5	3.9	4.7	29	7.2	8.8	11.0
5	2.8	4.0	4.7	30	7.3	9.2	11.4
6	3.7	4.7	5.9	31	7.6	8.9	10.8
7	3.9	4.2	5.9	32	7.8	9.2	11.6
8	4.1	5.2	6.2	33	7.8	9.2	11.6
9	4.6	6.0	7.4	34	7.8	9.5	11.0
10	4.9	6.0	7.5	35	8.2	9.7	11.6
11	5.0	6.4	7.8	36	8.4	9.8	12.0
12	5.0	6.2	8.0	37	8.4	9.9	12.1
13	5.0	6.4	8.0	38	8.5	9.9	13.0
14	5.1	6.5	8.3	39	8.5	9.7	11.7
15	5.2	6.5	8.3	40	8.6	10.3	12.6
16	5.9	7.4	9.2	41	8.6	9.9	12.0
17	6.1	7.7	9.4	42	8.6	10.0	12.2
18	6.3	8.0	10.0	43	8.8	10.0	12.5
19	6.3	7.7	9.8	44	8.9	10.2	13.0
20	6.4	7.8	9.7	45	8.9	10.2	12.2
21	6.4	8.1	10.0	46	9.2	10.5	12.7
22	6.4	7.7	9.6	47	9.3	10.6	12.2
23	6.5	8.1	10.0	48	9.4	10.8	13.0
24	6.6	7.9	10.1	49	9.6	10.8	13.0
25	6.7	8.4	10.5	50	9.7	11.0	13.2

LIFE HISTORY AS DETERMINED BY ANALYSIS OF DISTRIBUTION CURVES

By examination of the distribution curves, figures 1 to 12, it is at once apparent that there are two distinct size groups in each population. Each graph by its bimodal character gives conclusive evidence of this fact. This was first interpreted to mean that the animals might have a life cycle of two years, the first mode (from left to right) representing

the young produced during one year, and the second mode, or adult group, that of the preceding year's reproduction. Slight irregularities in the curve between these two major modes were interpreted as being due to seasonal variations or to a protracted and interrupted breeding season. This supposition was adhered to during the greater part of the study and was discarded only after it was discovered that each individual bears two broods in each year which thus impress two modes upon the graph for each population.

It was obvious from the comparison of graphs made for each month that growth was taking place in the members of each size group. This was more apparent in the young than in the adult. Throughout the course of the investigation it was apparent that the members of this species have a very definite limitation of maximum size. Soon after this maximum is attained the individual dies. Proof of this was attested by the fact that individuals of maximum size for this species were wanting in some of the monthly samples and the further fact that dead shells of the largest size groups were encountered in field collections in greater numbers than for any other size groups. No doubt the period of senescence which the larger adults have approached or are approaching has its effect on the modification of the growth rate.

The cumulative effect of mortality from natural causes is reflected in the relative height of the two modes for many months (figs. 1 to 5). As might be expected, the juvenile generation in each population exceeds the numbers of the adult generation except in the months of August and September (figs. 7 and 8) when the period of reproduction is reduced to the minimum. In July (fig. 6), the adult generation has almost wholly disappeared, but by November (fig. 10) the juvenile generation has become adult and a new brood of young has begun to appear in significant numbers. In figures 13 and 14, the progressive advances of the modes of the coexisting generations in each population are shown graphically by superimposing the June graph upon the one for March (fig. 13) and the December graph upon the one for September (fig. 14).

Intimate field and laboratory study was suspended during the summer months, but collections were taken each month as before. On resuming the study of materials in the fall, and tracing the growth rate, it became apparent that the young group had attained the same general place in the size range that featured the supposedly two-year-old group of individuals of the first collections. A new 'young' mode appeared in the August curve (fig. 7).

Observations of numbers of young in May, June, and July collections were also at variance with a preconceived belief founded on reports of other workers studying members of this family. These facts led to a search for means of determining the age of this species. Growth rings on the shell have been used for some time in determining the age of the Unionidae. Gilmore ('17, p. 27) discusses the possible interpretation of age in the Sphaeriidae by means of growth rings. "The distinctness of growth areas or rings in *Sphaerium* led to the belief that age might be determined by correlating size and number of rings. The entire surface of the shell is thrown in parallel ridges which represent growth lines. At the beginning of a season these lines are widely separated; at the end they are crowded very close together. One of these areas constitutes a growth ring." He measured 400 specimens of *Sphaerium* simile with varying results and makes no definite conclusions except that such ring does not necessarily indicate a season's growth. He leaves the topic open with the following: "If one ring represents a year's growth, the average of a large specimen would be four or five years."

An attempt was made to use growth rings as an indicator of age in *Sphaerium solidulum*. A representative number from a single collection were examined to ascertain the number and character of the rings. Casual organo-leptic examination failed to disclose these areas in this species. A microscopical examination whereby the distance between lines was measured failed to disclose the areas so apparent in other species. However, concentric lines are prominent espe-

cially in the young of this species (personal communication of Doctor Sterki). It was thought that these might be used as a substitute for growth rings. However, the number of lines on the shells of the same size are so variable and irregular that this method would not serve as a basis for age determination. At least in *Sphaerium solidulum*, the lines incised on the shell have no value in determining age. In fact, conclusive evidence is produced later in this paper to prove that the individual life span is approximately one year, hence growth rings in the sense of annual rings are wholly wanting. Color bands on the shell at times produce zones that might be mistaken for growth rings, but all the facts in the case support the view that these color bands are accidental, probably produced by some change in the physical environment not correlated with growth.

While this phase of the problem was being considered, the collections of the year were completed. It was the amassing of these data in a single table (table 2), correlated with the field and laboratory observations of each sample, that finally led to a means of determining the age in this species and furnished a clue to the reproductive habits.

An examination of these data reveals that adult individuals of the maximum-size groups (contained in last four columns, table 2) are practically absent from the collections of August to February. This evidence is supported by the presence of many dead shells in the field collections. This was so noticeable that, while not even being considered as a significant factor during the course of the field collecting, observations were included in the field notes. If these animals had a life cycle covering two or more years, with reproduction occurring throughout the year as these observations indicate, and growth is continuous, there would be no collection in which individuals of the maximum-size group were absent. This is particularly true where each collection contained so many individuals. This evidence supports the view that this species has a relatively short life span of approximately one year.

TABLE 2
Showing the number of individuals of S. solidulum taken in each quantitative monthly collection and distribution according to size groups

Thickness, in mm.	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0
2/ 8/30	6	47	82	82	22	22	24	29	24	49	61	32	9	6	8	3	2	0
3/17/30	8	47	82	106	41	24	23	18	10	36	22	17	4	3	0	1	1	0
4/13/30	3	27	24	113	37	33	16	20	18	26	35	31	3	2	5	4	1	0
5/10/30	0	34	110	189	111	200	115	64	30	40	65	67	18	11	6	6	4	0
6/12/30	0	32	87	69	77	96	65	131	92	26	15	48	26	9	7	3	1	0
7/10/30	0	34	57	93	80	60	38	54	32	38	23	16	12	5	3	2	1	1
8/15/30	11	36	22	27	49	102	95	127	131	138	47	44	34	6	1	1	0	0
• 9/21/30	13	43	18	19	42	84	84	99	86	94	26	24	11	6	0	0	0	0
10/15/30	8	43	53	42	33	26	22	23	26	28	20	9	12	9	0	0	1	0
11/16/30	15	32	30	8	16	24	38	38	32	59	34	21	20	3	2	0	0	0
12/20/30	33	88	87	19	7	18	24	29	23	43	32	13	11	9	3	1	0	0
1/10/30	35	115	86	33	13	23	32	43	39	71	42	21	16	10	1	0	0	0
Total	132	578	736	800	528	714	576	675	543	648	422	343	176	79	36	21	12	1
Grand total, 7022.																		

However, the question of the bimodal nature of the curve still remained. The solution of this phase of the problem was obtained by a comparative study made by superimposing the distribution curves of one month upon those of succeeding collections. This study (figs. 13 and 14) revealed that there are two periods of accelerated reproduction in the year, one in late summer, and the other in the winter and early spring months.

There are at least two possible explanations of this phenomenon, namely, that there are two types of animals in the same species each producing one generation a year but at different seasons, or, that members of this species have two reproductive periods in their short life span of a year. The first alternative is not an impossible one, as it is a condition found among other animal groups. However, subsequent investigations of the marsupial pouches offered proof that each individual of this species has two reproductive periods in its life cycle. The results of this part of the study and conclusions formed from the evidence secured are more fully discussed in the section on 'Breeding habits.'

Collections from other sources indicate a high degree of correlation in results secured. Figure 18 presents a comparison of material representing two species taken from different sources on the same day. Though different species were concerned, the results are highly significant. The only sharp deviation in the correlation of the two distribution curves occurs in the size group that should have appeared in August. The sharp decline of numbers of young was undoubtedly influenced by a protracted drought which caused the stream from which one sample was taken to be reduced to a mere fraction of its usual flow, while conditions in the ox-bow pond were relatively constant. No doubt this factor modified the normal reproductive habits of the species living in the stream. The correlation is as close as would be expected of two samples from different habitats on the same day.

BREEDING HABITS

Data secured from this series of collections confirm the work of Drew ('95, p. 180) when he states: "Reproduction goes on during the greater part of the year." Gilmore ('17, p. 20) comments on the results of his study of a member of this family with the following: "The breeding season probably continues through the greater part of the year. Observations on this point has been very meager. . . . considerably over fifty adult specimens have been sectioned. All were found to contain young in several stages of development." The specimens that he examined were taken in November and December. According to information derived from the present study, his specimens were probably taken just at the beginning of the maximum reproduction period. Monk ('28, p. 496) from a preliminary study of the life history of *S. notatum*, made in connection with his anatomical studies, records: ". . . . that reproduction is at the maximum during the summer months and that very few are born during the winter. Conditions in the drainage ditch have been very unusual during the past year. There have been many protracted periods of high water, with a large amount of wash resulting, following heavy rains." Although the results of this study are in part contradictory to the results obtained by Monk, the factors mentioned by him may have caused the deviation from these findings, also his conclusions were based on observations of specimens kept in aquaria during the winter. It is to be remembered in comparing the two results that the species are different. I am of the opinion that there is as great variation in the life cycle in members of this family as exists among fishes or in any other much-studied group. Chamberlain and Jones ('29, p. 31) state: "The breeding season probably continues most of the year." This study indicates that the breeding season does continue throughout the year, but a preponderance of young are liberated during the winter months (table 2). This is contrary to the generally accepted belief that maximum reproduction obtains in the summer months. Following the winter months

there is a period of minimum reproduction that ends with the July collection. Subsequent monthly collections reveal another phase of increased reproduction in the August collection. This one is impressed on the distribution curves as a major mode referred to before as apparent in practically all of the monthly distribution curves.

An examination of the marsupial pouches of all the adults of the July and December samples indicates two reproductive periods for each individual of this species. This is not the first report of the number and size of embryos found in the marsupial pouches of the Sphaeriidae. Gilmore presents several tables ('17, p. 24) indicating numbers of young borne by a species of this family, also the size of the individual, both adult and embryo, are ably set forth. His conclusions are as follows: "The size of the young was found to be independent of the size of the parent. An eight-millimeter young is as likely to be in a fourteen-millimeter parent as of an eighteen-millimeter parent. The majority of the above contained two young, one in each gill. A few contained four." Frank C. Baker ('28, p. 307) records results, in tabulated form, of an examination of specimens of *Sphaerium striatinum* collected in early August. His table compares favorably with the results obtained in this study, although too few were examined by him to make conclusive comparisons.

Microscopic examination of the marsupia of all the individuals in every size group for which any evidence of maturity presented itself, established some pertinent facts on the reproductive habits of this species. In all, the marsupia of 230 individuals in the July collection and 188 individuals of the December sample were examined. The results of this investigation can be readily followed in the graphs (figs. 16 and 17). These were made in the same manner for the study of the embryos as the monthly quantitative collections, with the slight variation in that lengths were used to establish size groups instead of thickness. It was necessary to make this deviation from the preceding method because

the lengths of the embryos were obtained by the use of a compound microscope with the aid of an ocular micrometer. It would have been difficult to maintain the embryos in position on the slide to secure thickness measurements. However, this slight deviation in no manner affects the results secured. As shown earlier in this paper and in table 1, there is a very close correlation between length and thickness of *S. solidulum*.

The smallest individuals of *S. solidulum* found living free in nature were 1.5 mm. through the umbones. The largest marsupial young of this same species observed during the course of this investigation were 1.9 mm. Since there is no discontinuity in size between the marsupial young and the smallest independent young specimens, we have a positive check on the validity of the sampling of the populations. There is no break between the marsupial and free-living young. Furthermore, the growth of the young within the marsupial pouches may be projected as a direct backward continuation of the growth for the free-living population.

By an inspection of figure 16, which is the distribution curve of the embryos taken in the July collection, it is noted that there is a large percentage of embryos approaching the maximum size of the marsupial young. This indicates that a period of extrusion is close at hand. In the December collection there are no embryos of maximum length, although some are approaching it. This indicates that a large percentage of young have been extruded. This conclusion is confirmed by examining the distribution curve for December (fig. 11). This graph has an exceedingly high mode for the new brood which had been born, leaving the marsupia devoid of fully formed young.

An examination of the total of 418 parent individuals reveals that there are two distinct size groups in embryonic development. This is more apparent in individuals approaching the maximum adult size. In all instances where four embryos obtained in the pouches, two were distinctly larger than the remaining two. It is obvious that where such a

diversity of size occurs, there is only one logical conclusion to be reached. There must be two distinct periods of reproduction for each individual. Since evidence has been set forth earlier in this paper indicating that individuals of *Sphaerium solidulum* live approximately one year, these two periods of liberation must come within the same year. There are ample evidences to substantiate this conclusion. This is most convincingly borne out by the nature of the graphs of the embryos. The same bimodal character (fig. 16) which marks the marsupial young into two size groups is projected into and retained throughout the lifetime of the individuals, imposing upon the adult population (figs. 1 to 12) the same bimodal distribution conceived within the two-brood condition of the marsupial young. This two-brood condition must not be confused with that which is commonly referred to in works on the life history of insects, for in that instance the members of one brood are the parents of the succeeding brood.

Although the study revealed some instances of variation from the characteristic two or four embryos in the pouches, the variation is not regarded as significant. By far the larger number of individuals examined contained either two or four, that is one or two in each of the two pouches. The strict bilateral symmetry of the animals in all other respects indicates that such would be the case. In instances where three embryos were found, there was evidence that one of the larger embryos had been recently extruded, and the remaining embryo was approximately the same size as the smallest borne in the other marsupium. Doubtless in some instances the presence of one or three embryos may have resulted from the failure of an egg to develop properly or may be due to pathological destruction of an egg or embryo.

Table 3 was prepared to show the results of the comparative study of embryos where the two or four condition exists. This offers evidence to support the conclusions formed by the study of the embryonic distribution curves (figs. 16 and 17). Where the four embryos are present in the individual,

it is at once apparent from the table that this number is so small as to be almost negligible in the July collection, whereas the December collection shows a preponderance of this condition, particularly in the larger adult groups. On the other hand, the same sort of comparison of the two-embryo condition indicates a direct reversal of the first comparison. The two-embryo condition is found in the greatest proportion of the population in July. It is natural to conclude from the facts thus shown that there are two reproductive periods in the life cycle of the individual. This may be summarized in

TABLE 3

Showing correlation of size of parent and number of marsupial young in adults of S. solidulum collected in July and December

SIZE OF INDIVIDUAL	4-EMBRYO CONDITION		2-EMBRYO CONDITION		BARREN CONDITION	
	July	December	July	December	July	December
4.5	0	0	0	9	38	15
5.0	0	0	3	24	51	5
5.5	0	10	10	12	22	1
6.0	0	22	22	21	16	0
6.5	3	22	16	9	4	1
7.0	4	3	12	7	0	3
7.5	1	10	11	1	0	0
8.0	1	8	3	1	1	0
8.5	2	2	1	1	0	0
9.0	0	0	2	1	0	0
9.5	0	0	0	0	2	0
10.0	0	0	0	0	1	0

the following manner: the young produced in the winter months attains sexual maturity and produces its 'first brood' in August and its 'secondary brood' in the winter months, after which there is a period of senescence and finally death. The young born in the summer months attains maturity in the winter, producing its 'first brood' at that time and the 'secondary brood' in the late summer to complete the life span.

If this conclusion is true, there should be in the July population a mixed assembly of adults, those just attaining sexual maturity bearing two embryos and those of the mature adults

that have reached their final reproductive period bearing only two embryos or are barren. The summation of the studies as presented for that month confirms this viewpoint. The 'summer born' attaining sexual maturity produce their 'first brood' in the winter and reach mature adult stage in the summer following. On the other hand, the 'winter born' attaining sexual maturity in the summer months, reproduce their 'first brood' probably under adverse conditions, and, reaching the mature adult stage in the winter, the period of reproduction being favorable, contribute to the maximum numbers of young present. There is no evidence of cessation of reproductive activity at that time. The four-embryo condition so prevalent in the December collection is confirmatory of this conclusion. What factor or combinations of factors may be responsible for the period of maximum reproduction of this species coming in the winter months has not been determined in this work. Further investigations are in progress to determine, if possible, the cause of this phenomenon.

Figure 15 is a comparison of the collection taken in February of 1930 and that of 1931. A very close correlation of these two graphs indicates that the yearly cycle of the animals as exhibited by the distribution curve is being repeated and discounts any argument that methods and results are accidental.

Attention is called to the fact that while some of the conclusions reached may at first appear directly opposed to the general belief built up on the work of careful investigators, in every instance the data secured and the conclusions formed can be superimposed on similar investigations and help to explain some of the problems that confronted some of those workers. Gilman's work with this phase of the problem (life history and reproduction) is more understandable as to variation in number and size of embryos found in adults, when considered with the results of this study. His statement, "The size of the young was found to be independent of the size of the parent," is explained on the basis of two reproductive periods in the year for each individual. Ma-

terial taken at random would tend to show the results he secured.

The adults of maximum-size groups of *S. solidulum* were all sterile in specimens examined. Monk ('28) indicates in his preliminary survey of the subject that the same conditions prevail for *S. notatum*. "... in animals killed in early July, the germ follicles are much shrunken and very nearly empty." This, in the light of this study, means senescence of the individual. Another part of his observations is confirmed by: "During the first week of August, it was observed that the full-grown animals were giving birth to but two young, while the young adults from 9 to 11 millimeters in length, were giving birth to quite a few." This is explainable on the basis of two reproductive periods for the individual. The young adults were of the 'winter born' group just attaining sexual maturity, while the adult forms had reached senescence.

GROWTH

Observation on the progression of the modes from month to month are best shown in figures 13 and 14. The gradual progression of the modes through successive monthly periods indicates that individuals which at birth are approximately 1.5 mm. in thickness (about 4 mm. in length) in ten to twelve months reach maximum size of 10 mm. (slightly over 13 mm. in length). Expressed in increments of thickness, this would mean an average growth of approximately 0.5 mm. per month. In graph 13 we have fairly conclusive evidence that the mean for the small individuals of the population has advanced 2 mm. in a period of three months, while for the larger individuals the mean has advanced only 1 mm. in the same time.

SUMMARY

1. Twelve monthly quantitative collections made from the same habitat making 7022 individuals, or an average monthly collection of 585 individuals, of the same species (*Sphaerium solidulum*).

2. Distribution curves were prepared based on numbers of individuals falling into eighteen size groups established upon thickness of animal.

3. Correlation of size groups of material taken from different habitats is noted.

4. No individuals of maximum-size groups are present during months from August to February.

5. Individuals of the species under observation have a distinctly limited life span of approximately one year.

6. The analysis of the distribution curves of each quantitative sample indicates two size groups in each collection as shown by the bimodal character of each graph.

7. The period of maximum reproduction of this species does not occur in the summer months, as previously believed, but in the winter months.

8. Seasonal growth rings are not present on this shell, although concentric lines are characteristic.

9. Anatomical investigation of marsupial pouches of the December and July adults reveals: a) Marsupial pouches generally contain two or four embryos. b) When four obtain in pouches, two are distinctly larger than the remaining two. c) Embryos are found in individuals of size groups that have attained one-half maximum adult size. d) A larger percentage of 'young' adults appears in December than in July. e) Distribution curves of embryos duplicate the bimodal character of the young and the adult.

10. Maximum-size adults are apparently sterile.

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EXPLANATION OF PLATE I

Showing individuals of *S. solidulum* taken from the eighteen size groups established on the basis of thickness of the animal. One was taken from each size group ranging from 1.5 to 10 mm. in thickness through the umbones. The individual in the lower right-hand corner is one selected from the 8-mm. group to show the relation of thickness to length.

THE LIFE HISTORY OF SPHAERIUM
THURAL DALE FOSTER

PLATE 1

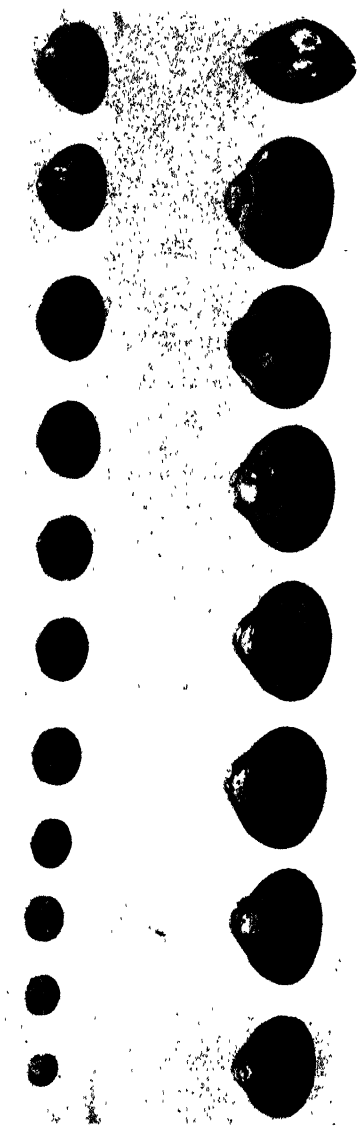


PLATE 2

EXPLANATION OF FIGURES

Twelve graphs showing distribution of individuals of *S. solidulum* according to thickness through the umbones. In each graph the number of individuals has been reduced to a common basis of 1000 to make direct comparison. The increments on both axes are uniform throughout figures 1 to 12.

- 1 Graph showing distribution of 508 individuals collected February 8, 1930.
- 2 Graph showing distribution of 443 individuals collected March 17, 1930.
- 3 Graph showing distribution of 398 individuals collected April 13, 1930.
- 4 Graph showing distribution of 1070 individuals collected May 10, 1930.
- 5 Graph showing distribution of 786 individuals collected June 12, 1930.
- 6 Graph showing distribution of 550 individuals collected July 10, 1930.
- 7 Graph showing distribution of 871 individuals collected August 15, 1930.
- 8 Graph showing distribution of 649 individuals collected Sept. 21, 1930.
- 9 Graph showing distribution of 355 individuals collected October 15, 1930.
- 10 Graph showing distribution of 372 individuals collected Nov. 16, 1930.
- 11 Graph showing distribution of 440 individuals collected Dec. 20, 1930.
- 12 Graph showing distribution of 580 individuals collected January 10, 1931.

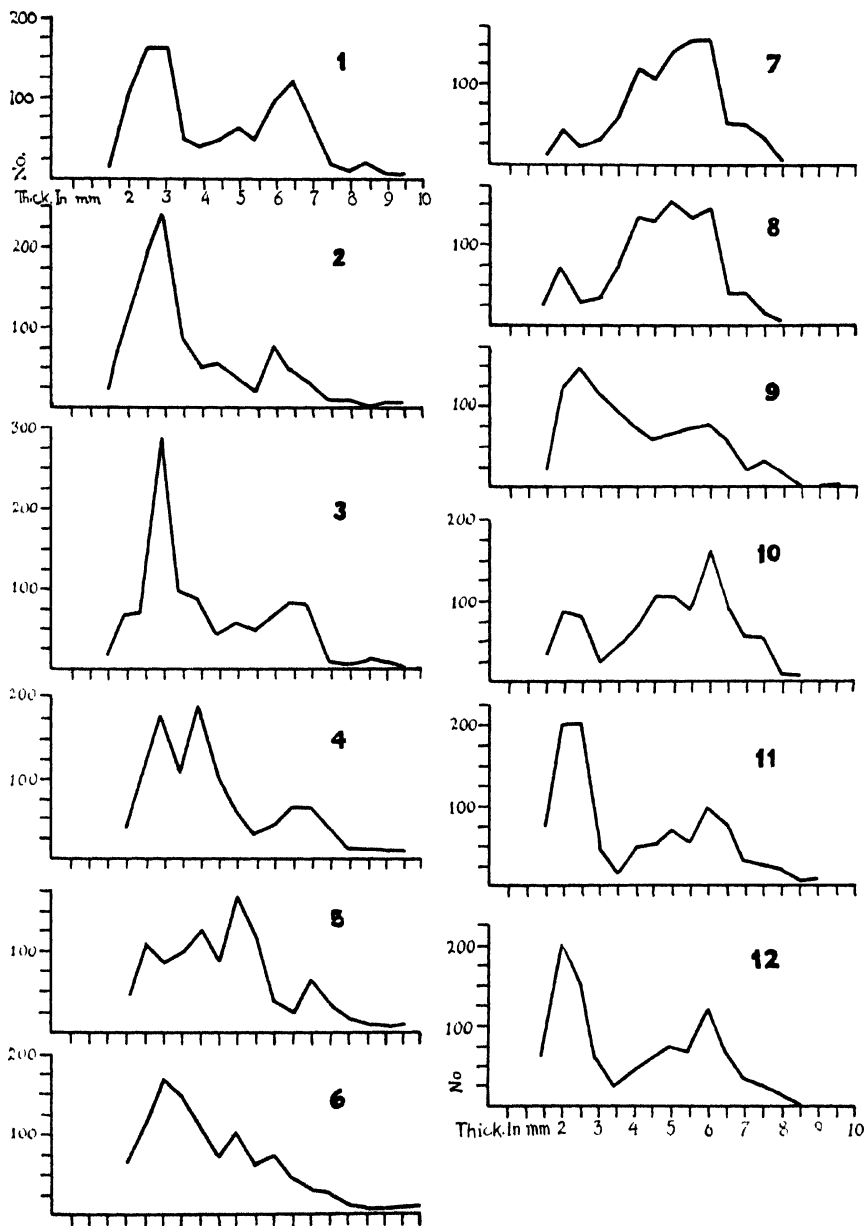


PLATE 3

EXPLANATION OF FIGURES

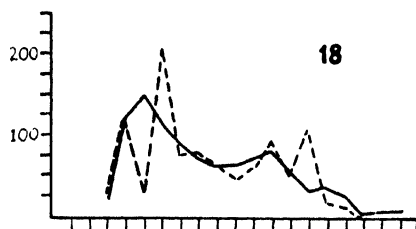
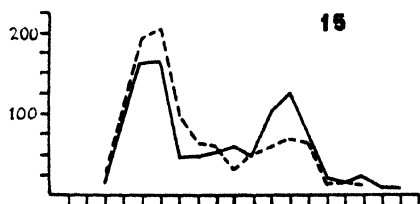
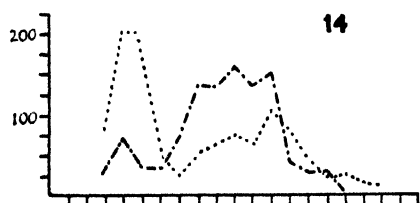
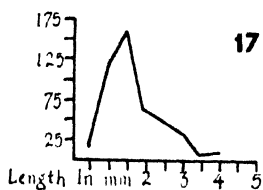
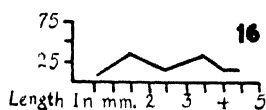
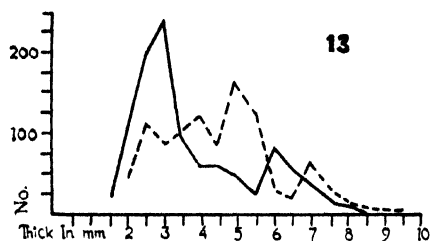
13 and 14 Graphs showing the progressive advances of the modes of coexisting generations in each population by superimposing the June graph (broken line) upon the one for March (solid line), figure 13, and the December graph (dotted line) upon the one for September (dash-and-dot line), figure 14.

15 Graph showing the correlation of the distribution curves of *S. solidulum* taken February 8, 1930 (solid line) and February 8, 1931 (broken line).

16 Graph showing the distribution of embryos, according to size, found in all adults of *S. solidulum* taken in the July collection.

17 Graph showing the distribution of embryos, according to size, found in all adults of *S. solidulum* taken in the December collection.

18 Graph showing the correlation of the distribution curves of *S. solidulum* (solid line) with an unidentified species of the same genus (broken line) taken from different habitats on October 15, 1930.



STUDIES ON THE LIFE CYCLE OF THE SNAIL, VIVIPARUS CONTECTOIDES¹

HARLEY J. VAN CLEAVE AND LUDWIG G. LEDERER

TEN FIGURES

AUTHOR'S ABSTRACT

More than 3700 individuals of *Viviparus conetectoides* from the Illinois River, near Peoria, Illinois, and from the Erie Canal in central New York, have been the subject of statistical and biological analysis as periodic quantitative samples to secure information on the life cycle. Sexes are easily distinguished through tentacular differences. Shells show marked sex dimorphism of size. Males reach a maximum height of 25 mm.; females may exceed 40 mm. Distribution curves, checked by experimental data on another species of the same genus, give evidence that males live normally about one year, while females may live three years. Differences in sex ratio are attributable to differences in life span and to changes in environmental factors producing aggregations of members of one sex.

In central New York, the parturition period seems to begin to March and terminates in June. In central Illinois it extends from February into May. The uterus of a gravid female contains eggs, embryos, and shelled young in a graded series. At birth, the young shell contains one and one half whorls and has a height of approximately 3.8 mm. Color bands are present on the shell three months before birth. Initial growth rate is extremely rapid. In less than three months the largest of the new generation are as large as the smallest of the parent generation. Graphs given show seasonal changes in the population.

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¹ Contributions from the Zoölogical Laboratory of the University of Illinois, no. 428.

INTRODUCTION AND ACKNOWLEDGMENTS

The genus *Viviparus*, represented by a large number of species, is almost world-wide in its distribution. All the members of this genus give birth to fully formed young, but specific details as to the exact method and time of reproduction, rate of growth, data concerning the sex ratios, and other significant biological facts pertaining to the two sexes are lacking for all but a very few of the species. Of the species which inhabit North America, there is no specific information available on any aspect of the reproductive and growth cycles.

Viviparus contectoides, with its robust shell bearing four conspicuous brown, spiral bands, is the most characteristic North American representative of the genus. It is widely distributed in lakes and streams of eastern United States and has been the object of study for more than a century. At first it was thought to be identical with a European species, until Binney, in 1865, pointed out the fact that the American shells have four color bands, whereas but three are found on their European relatives. In spite of a long nomenclatorial history, the conspicuous appearance of the shells, and the unusual birth habits in this species, no one has ever published a significant contribution to its biology.

The authors are especially indebted to Mr. Frank C. Baker, not alone for confirmation of the identification of the materials upon which we have worked, but even more for the personal interest which he has manifested in the unfolding of the problem and for the valued information and advice coming from his long contacts with molluscan problems and literature. Philip and Dorothy Van Cleave have aided in the collecting of specimens, in segregating the sexes, and in tabulating the results of the observations taken in the summer of 1931. Bernice F. Van Cleave has also aided in the above-mentioned tasks.

MATERIAL AND METHODS

Localities

During the summer of 1930, the senior author, while engaged as field naturalist for the Roosevelt Wild Life Station, discovered an especially favorable habitat for *Viviparus contectoides* in the Erie Canal, at Durhamville, New York, and secured a quantitative sample for statistical and biological analysis. This collection, and other materials from the Illinois River in the vicinity of Peoria, Illinois, were studied by the junior author, who took measurements of height and diameter of 902 shells, determined the sex, and subjected the material to statistical analysis under the direction of the senior author. The collections and field observations of the summer of 1931 were made by the senior author, who also completed the analysis of the data and prepared the manuscript. In the progress of the present study, more than 3700 individuals of *Viviparus contectoides* have been subjected to measurements and analysis. Nearly 3000 of these were taken from western New York during the summer months. The approximately 700 specimens from the Illinois River covering the remainder of the year furnished the basis for a more comprehensive understanding of the species than might be appreciated at first glance. The relatively small number of shells collected from the Illinois River served for the determination of the rate of growth of the marsupial young and the more exact fixing of the limits of time of parturition just as satisfactorily as could have been determined by more extensive series of individuals.

A tabulated summary of the collections used in this study is given in table 1.

Method of sampling the population

Most of the collections have been quantitative samples of the population taken in such manner as to avoid selection of individuals of any particular size or condition. When snails were plentiful, the limits of a given area of stream bottom or

of stream margin were marked off and then by repeated use of a dipnet the prescribed area was dredged repeatedly to insure securing all of the snails of all sizes. Though practically all of the specimens were on the bottom and clearly exposed to view, the surface layer of approximately one inch

TABLE 1

Sources of collections and proportions of the sexes in Viviparus contectoides

DATE	SOURCE	COLLECTOR	♂♂	♀♀	RATIO ♂♂ TO ♀♀	TOTAL
July 20, '30	Erie Canal, Durhamville, N. Y.	Van Cleave	..	..		79
July 26, '30	Erie Canal, Durhamville, N. Y.	Van Cleave	54	74	1: 1.37	128
Aug. 28, '29	Short Point Bay, Oneida Lake, N. Y.	Van Cleave	..	..		8
Nov. 9, '30	Illinois R., Mill Point, above Peoria, Ill.	F. M. Hunt	28	94	1: 3.36	122
Feb. 2, '31	Illinois R., Upper Peoria Lake	Lederer	268	305	1: 1.14	573
Feb. 27, '31	Illinois R., Upper Peoria Lake	Lederer	..	..		25+
May 9, '31	Illinois R., Upper Peoria Lake	Lederer	..	..		25+
June 17, '31	Erie Canal, Durhamville, N. Y.	Van Cleave	142	190	1: 1.34	332
July 17, '31	Erie Canal, Durhamville, N. Y.	Van Cleave	302	406	1: 1.34	708
July 27, '31	Erie Canal, Durhamville, N. Y.	Van Cleave	238	195	1: 0.82	433
Aug. 16, '31	Erie Canal, Durhamville, N. Y.	Van Cleave	267	275	1: 1.03	542
Aug. 23, '31	Erie Canal, Durhamville, N. Y.	Van Cleave	119	33	1: 0.28	152
Aug. 31, '31	Erie Canal, Durhamville, N. Y.	Van Cleave	227	355	1: 1.56	582
Total,						3709+

of mud and silt was sieved methodically to avoid the possibility of losing some of the smaller snails. In midsummer the snails tended to form dense aggregations along the margins of the Erie Canal in the submerged, fixed vegetation and the algae. Under these conditions, a representative collection of approximately 500 snails could be taken from a few

square yards of bottom in water not exceeding a foot in depth. Because of the abundance of *Viviparus* in this habitat, it became readily possible to take large representative collections at intervals of about two weeks, allowing a broad enough space between collecting sites to avoid the complications of depopulation and repopulation of a given area. All of the larger, older shells in the Erie Canal were heavily overgrown with a coating of filamentous algae, a fact which precluded the possibility of securing good photographs of the snails in this habitat.

Because of the adverse conditions of low water, combined with heavy sewage contamination, the collections from the Illinois River were obtained only with the greatest difficulty during the winter and spring of 1930-1931. Under these circumstances, the living snails had migrated into water four or five feet deep. This marked response to seasonal or environmental changes in the habitat is probably one of the reasons why *Viviparus* becomes so readily adapted when introduced into a new situation. This same facility of response is called upon later in this paper to explain abnormal conditions in some of the samples that had been thought to be representative.

ANALYSIS OF THE POPULATION SAMPLES

Throughout this series of studies, the first step in the analysis of each collection was the elimination of all dead shells, for only the living snails give a true picture of the population and yield data from which growth curves may be constructed. In one collection only were the dead snails taken into account and in this instance the snails had died so recently that the bodies were still contained within the shells and were available for the determination of the sex and observations on the marsupial young.

In the Erie Canal, the areas under investigation were practically pure populations of *Viviparus contectoides*. The collections from the Illinois River were in most instances mixed populations of *Viviparus*, *Campeloma*, and *Lioplax*. Care

was taken to eliminate all contamination of other species from the samples.

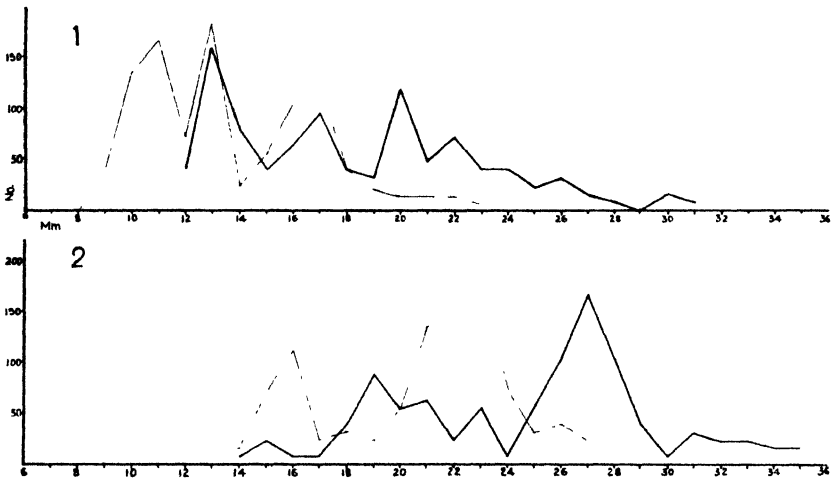
A vernier caliper was used for all the measurements. In the early part of the study, two measurements, height and diameter, were taken for each individual. All measurements were recorded in tenths of a millimeter, but a preliminary analysis of the data revealed the fact that intervals of one millimeter were adequate for depicting the rate of growth and significant size differences between natural groups. In preparing the graphs, the data for each collection were corrected to the common basis of one thousand in order to facilitate direct comparisons between the various collections.

Sexual dimorphism

Many collectors of *Viviparus* have been unable to determine the sex of the individuals in their collections. Baker ('28, fig. 14) has very clearly figured the genitalia of the male of one species of *Viviparus*, and Binney, many years earlier ('65, figs. 30 and 31) gave sketches for the differentiation of the sexes in *V. intertextus*. Most collectors have probably expected to find males among the large specimens which usually find their way into cabinets. In the course of the present investigation it was discovered that there is marked sexual dimorphism of size in *Viviparus contectoides*. While a single female shell over 40 mm. in height has been encountered in our collections, we have never found a male of this species exceeding 25 mm.

In so far as the authors have been able to determine, there is no constant character of the shell for differentiation of the sexes. In the preliminary studies, it was hoped that there might be some differential between height and diameter correlated with the sex of the animal. The close correlation between height and diameter for the entire population curves (figs. 1 and 2) demonstrated that no basis for sex recognition resides here, so in all of the later studies the single measurement of height was taken.

In the male of *Viviparus*, the right tentacle is conspicuously heavier than, and of a shape different from, that of the left, for it serves as an intromittent organ through the tip of which the vas deferens opens. It is necessary to compare the two tentacles before the sex may be recognized. For segregating living snails, it was found that the tentacles are readily observable when the animals are placed in shallow



Figs. 1 and 2 Graphs showing correlation of height (heavy line) and diameter (light line) in populations of *Viviparus contectoides*.

Fig. 1 Distribution in a typical population of *Viviparus* from the Erie Canal at Durhamville, New York, in midsummer (July 26, 1930).

Fig. 2 Distribution of a typical population of *Viviparus* from the Illinois River near Peoria, Illinois, in late fall (November 9, 1930).

pans with just sufficient water to cover the shell. As the heads are protruded the sex is readily determinable, for the blunt, thick right tentacle of the male stands in sharp contrast to the fine-pointed left tentacle and presents an asymmetry never encountered in any female of the species. This means of separation of the sexes was found applicable even for the newly born individuals less than 4 mm. in height. By keeping several pans under observations simultaneously, a collection of 400 snails was segregated in less than an hour's

time. Similar samples, segregated by the alternative method of dissecting out the preserved bodies and uncovering the tentacles, required about five hours of tedious dissections under a binocular.

In all of the large females collected before the close of the reproductive period, additional proof of the sex was available in the form of a conspicuous marsupial pouch filled with developing eggs or fully formed young snails which are readily distinguishable through the walls of the pouch. In the collections taken in midsummer (July in western New York), there were no marsupial young bearing shells. Some individuals at this season bore relatively large eggs which were easily recognizable upon rupture of the marsupial chamber. The dimorphism of the sexes, as reflected in relative size of the shells, was discovered early in this investigation. In the preliminary studies on the recognition of the sex of preserved specimens, the examinations were first confined to the largest individuals, since in them the eggs and fully formed young were so readily found. Following this procedure, all specimens more than 25 mm. in height had been examined before a single male was encountered (figs. 6 to 8). All subsequent observations gave confirmation of the fact that in *V. contectoides*, in the habitats under investigation, the male never exceeds a height of 25 mm.

Sex ratios

There is wide divergence in the relative numbers of the two sexes in *Viviparus contectoides*. Similar divergence has been observed earlier by workers dealing with other species of the genus *Viviparus*. Thus Rao ('29, p. 280), in his work on the Indian *Viviparus bengalensis*, states: "The proportion in the numbers of male to female individuals is not the same in lots collected from different localities. In some places the males and females are equal in number, in others the females predominate." In our studies of sex ratios in *V. contectoides*, we have found corresponding variability in sex ratios even in collections from the same locality. This

variation we attribute to local environmental factors and to seasonal factors correlated with the life history rather than to locality differences as expressed by Rao in the above-quoted statement.

In extensive collections from a short stretch of the Erie Canal, the ratio of males to females varied between the extremes of 1 male:0.28 female, and 1 male:1.56 females. These differences do not reflect significant or actual differences in the relative abundance of the two sexes. Rather, they are the outgrowth of faulty sampling of the population. Significant differences in the same locality at different seasons of the year very obviously must exist, for, as will be shown later in this paper, there is marked inequality in length of the individual life span for the two sexes. In the Erie Canal, in midsummer, when most of the intensive study has been made, the ratio has been rather consistently between the extremes of 1:1.34 and 1:1.56. That other ratios for midsummer are unreliable indices is given evidence in the fact that since no reproductive period intervenes between late June and the end of August, any significant factor influencing the sex ratio would of necessity leave a permanent influence on it. If females had been reduced in actual numbers to produce the low ratio of 1 male:1.03 females on August 16th (see table 1), it would have been impossible for the females to again rise to a high point on August 31st (1 male:1.56 female). It is on evidence of this sort that the authors rest their claim that the sex ratio of a sample may be an unfair index to the sex ratio of the true population.

The two extremes of unusual proportions were from areas that were never collected over more than once. The ratio of 1 male:3.36 females, found in the sample from the Illinois River on November 9, 1930, gives evidence that the males of the older or parent generation die off in the fall or winter, leaving only the males born the preceding spring. In fact, the males of this sample in their distribution conform to a simple population curve giving no evidence of the survival of any of the parent generation. Some of the other devia-

tions from the usual ratio are not readily explainable. The dearth of females on two different dates at two different stations in the Erie Canal cannot possibly be a reflection of conditions having any significance in the life history of the species. In segregating the sexes of the sample taken on July 27, 1931, the senior author and his son independently observed that when the snails were placed in shallow pans of water, numbers of males crept from their shells and began active locomotion before a single female became active. This observation, made in advance of the discovery of the disproportionate number of males, is mentioned because of the possible light that it may throw upon the reversal of the sex ratio in this sample. In the field, when this sample was being gathered, it was noted that the snails were densely aggregated in unusually compact clumps massed in the vegetation zone in shallow water at the margin of the canal. The entire sample of 433 individuals was taken in a few short sweeps of the net in this weedy margin. Very few individuals were on the sloping bottom leading from this shallow water to the deeper channel of the canal. The very striking aggregation of the snails may have been in response to temporary alteration of the environment resulting in the establishing of more favorable conditions in the shallow water than maintained in other parts of the canal. With such a differential once established, the more rapid reactions of the males noted above could very readily lead to a segregation of the sexes, producing a temporary disturbance of the ratio of the sexes at a given point.

In the foregoing paragraphs it has been shown that sex ratios may vary widely in samples taken from the same body of water. Contrary to the statement of Rao, mentioned above, these differences are not correlated with locality, but lead to the conclusion that seasonal factors correlated with differences in length of the life span of the two sexes and one or more physical, environmental factors may operate singly or in combinations to produce differences in the sex ratio of samples of *Viviparus contectoides*. The differences grow-

ing out of the inequality in length of the life span are significant differences of biological import, but the ones emerging from differential in reaction to environmental factors have no significance in the interpretation of the life history.

From all the data before us, it seems that at birth the two sexes are present in equal numbers. By midsummer the longer life span of the females has the cumulative effect of producing a preponderance of females in the ratio of 1 male: 1.3 or 1.4 females. By late fall or midwinter the older males die off, leaving only the young males of the preceding period of parturition. By this elimination of males, the cumulative effect of simultaneous existence of two or three generations of females continues to magnify the advantage of the females in the ratio until there may be two or three females to a single male. This condition persists until the following spring, when the new brood, again with equivalent numbers of the two sexes, brings back the male ratio to the proportion characteristic of midsummer.

OBSERVATIONS ON THE LIFE CYCLE

Determination of the birth period

The analysis of periodic quantitative collections and field observations were combined to secure information on the period at which the young snails are liberated from the marsupial pouch of *Viviparus contectoides*. None of the females taken from the Erie Canal in New York State in June and July bore fully formed young. Only developing eggs, without shells, were in the marsupia of females taken during the early summer months. By late summer, some of the eggs had consummated their development and the embryos had attained the characteristic shell. A series of collections taken from the Erie Canal and from Oneida Lake, New York, in the month of August, contained females bearing marsupial young with conspicuous shells. All of the large females collected from the Illinois River in November bore widely distended marsupia, many of them packed with young snails. Field observations and collections from the Illinois River on

February 27th resulted in the discovery of gravid females which gave birth to normal young in less than twenty-four hours after removal to a laboratory aquarium and continued to discharge young over a period of five days. Against the possibility that laboratory conditions or handling might have induced premature abortion stands the evidence that measurements of young recovered from the Illinois River on May 9, 1931, added a normal continuation to the growth curve (fig. 4) depicting the increase in size of the laboratory-reared specimens through a period of seven weeks and four days of life in an aquarium.

Thus, in central Illinois, the females of *Viviparus contectoides* have a period of parturition beginning normally in late February and continuing for the species over a term of several weeks, since some females taken May 9th still retained fully formed young in the marsupium. The most active period of liberating young for this species seems to fall in the months of March and April in central Illinois.

Observations presented later in this paper indicate that in central New York this same species gives birth to young from some time in March until June. Judging from the growth curves, the largest number of young must be produced in April and May. This difference in parturition period of *Viviparus contectoides* in central Illinois and central New York is in general accord with other phenological observations in the two localities.

Not all of the young of a given female are discharged at one time. Laboratory experiments demonstrated the fact that they continue to be given off a few at a time over a period of several days. This observation is in keeping with the facts brought out later in this paper regarding diversity in extent of development of the young and embryos inward from the mouth of the uterus.

A careful examination of the females taken at different seasons of the year and the limitation of the appearance of young in the collections combine to offer proof that each year there is but a single season for the production of the

young in this species. This period extends over approximately three or four months, the exact dates varying with the relative advancement of the season.

In the present study, the writers have made no attempt to study copulation in this form and have not succeeded in observing copulation either in the field or in laboratory specimens. Since there is a period of several weeks following the act of parturition during which all females are non-gravid, it seems probable that copulation and fertilization occur soon after the brood is discharged, for with great regularity a new brood has filled the marsupium by late summer.

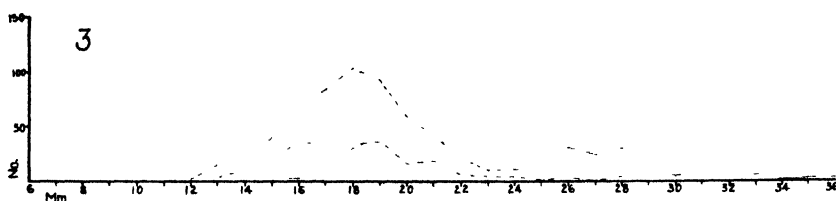


Fig. 3 Graph showing analysis of a population sample of *Viviparus contectoides* from the Illinois River on February 2, 1931, prior to the onset of the parturition period. Distribution of gravid females according to height of shell in solid line, males in broken line, non-gravid females in dot-and-dash line.

The marsupial young

During the period of gestation, the marsupial pouch of each mature female contains a graded assortment of eggs, embryos, and young in progressive series from the small posterior pocket down to the enlarged distal portion. Similar conditions have been observed in other species of the genus by Swammerdam (1758) and more recently in the Indian species, *Viviparus bengalensis*, by Annandale and Sewell ('21), and by Prashad ('28). The female bears her first brood when she is approximately one year old. There is some evidence that at least part of the females in the three-year group are sterile, for as shown in figure 3 many of the largest females were non-gravid when broods were present in the marsupia of smaller individuals.

The exact extent of the period of gestation for any individual female has not been determined experimentally. The fact that there is a single parturition period during the course of the year has been demonstrated by statistical analysis of the quantitative samples of the population. The spent condition of the females at the close of parturition in early summer gives evidence of a short period of uterine vacancy in the early months of summer. The establishment of the limits of the parturition period to approximately a span of three months in the spring gives evidence that between eight and ten months are requisite for the complete development of the egg to the time of birth. For at least six, and possibly seven, months of this uterine period the embryo is provided with a distinct external shell.

The smallest shells observed on the marsupial young of *V. contectoides* consisted of but one and one-half whorls. In progressive order down the uterus the number of whorls increases until three and one-half are present on the snails ready to be liberated.

Embryonic shells bear numerous setae arranged as spirals following the whorls. On the second whorl the setae are arranged in four spiral rows, which increase to six or seven on the last whorl. This is not in conformity with the observation of Rao ('28) in his statement that three rows of setae are characteristic of the embryonic shells of members of this genus. Setae persist and are clearly observable on the shells of some adult individuals. In the embryonic stages, the whorls are ridged, but the ridges are lost and the contour of the shell becomes smoothly rounded before birth.

Genesis of the color pattern

On the question of the origin of the color pattern upon the shell in *Viviparus* there has been marked lack of agreement in the published researches. Annandale and Sewell ('21, pp. 279 and 286) state that: "in those species in which the embryo has a comparatively large number of whorls before birth, the dark spiral bands characteristic of some such forms

begin to appear on the younger parts of the shell before it is set free from the egg membrane." This statement is in agreement with facts observed in the present investigation, for we have found that color bands are well formed on the shells of *V. contectoides* at least three months before birth. Shells having more than two whorls have a definite color pattern of three brown bands and even the fourth band, distinctive of this species, begins to appear before the time of birth.

The young at birth

The largest marsupial young at the height of the season of reproduction average about 3.8 mm. in height, though there is considerable individual variation from that size. The smallest free-living young taken from the natural habitat have exactly the same size as the fully formed marsupial young. When liberated from the marsupium, the shells which we have examined consist of three and one-half whorls. Individual variation in the size of the shells at birth combined with the relatively long period of production of young, yield a total range of only 6 mm. in the size of the young of the new generation at the close of the parturition period. This difference of 6 mm. between the smallest and largest young in the new generation is carried for a period of several months. There must be very little individual difference in rate of growth during the first few weeks of free life or there could not be such marked uniformity in the progression of the modes.

Height versus diameter

A preliminary examination of a large collection of *Viviparus contectoides* gave the impression that shells of this species vary widely in proportion of diameter to height. In an attempt to discover the basis for this impression, more than 900 individuals were measured with vernier calipers to determine both height and diameter (figs. 1 and 2). It was noticed that these shells are remarkably free from decollation. The small, apical whorl is clearly defined on even the largest

shells, hence the effect of decollation could not be summoned to explain the impression of variability in relation of height to diameter. An analysis of the results of the above-mentioned measurements gave proof that disparity in proportions is not due to individual peculiarities, but results from a conspicuous differential in the normal growth process. Shells of a given height have a consistent correlation between height and diameter, but newly born and marsupial young are markedly different from older shells in the ratio of diameter to height. The young shell is disproportionately broad, but the young *V. contectoides* grows much more rapidly in height than in diameter during its first year.

Because of this observed difference in rate of growth in height and diameter, when graphs based upon height and diameter of the same population sample are compared, successive modes for the two curves are not spaced equivalently. In a collection taken from the Erie Canal in July, 1930 (fig. 1), the first mode for diameters falls at 11 mm., while the first mode in the height curve occurs at 13 mm.—a difference of but 2 mm. between the two modes. The next mode in the curve for diameters is at 13 mm., while the corresponding mode for height is at 17 mm.—a difference of 4 mm.—which is again repeated in the distance between the next larger group for each of the two dimensions. This differential is not correlated with sex differences, for disparity between height and diameter in male and female shells is identical, and in both sexes is confined to the early months of free life. There is, therefore, a definite relationship between height and diameter in the shells of *Viviparus contectoides* of the same age. The initial disproportion between height and diameter which maintains at birth is rapidly compensated for by an early stimulus to growth in height. The young shell is not a miniature of the adult, for axial differences in rate of growth are sharply marked.

Rate of growth

Observations on aquarium specimens, checked by field notes, indicate that in the period of seven weeks from birth the young of this species have grown in height from 3.8 mm. to 7 mm. Starting free life with three and one-half whorls in the shell, initial growth is so rapid that an additional whorl is completed within the first three months after birth. By the end of the first year, the smallest individuals have reached the 15-mm. class, and most of the individuals are from 16 to

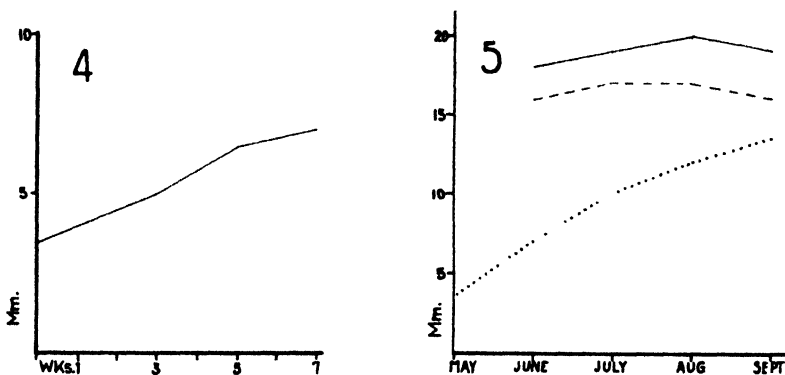


Fig. 4 Graph illustrating the rate of growth in height of the young of *Viviparus constrictoides*, from birth through the first seven weeks of free life.

Fig. 5 Graph showing growth in height of newly born individuals (dotted line) during the summer months. For comparison, the growth rates of one-year-old males (broken line) and one-year-old females (solid line) are superimposed on the same axes. Data compiled from distribution curves of quantitative population samples, not from growth of individuals.

19 mm. in height. The rapid initial growth in height is shown graphically when figure 6 is compared with figure 9, for in the interval of two months the mode for the new generation has progressed from 7 to 12 mm. In figure 5 this comparison of initial growth rate with rate of increase in the second year is even more clearly shown.

Length of the life span

Marked size dimorphism of the sexes adds complication to any interpretation of growth data. Until the sexes are

treated separately, conspicuous modes may signify either age or sex difference. Through most of the present series of studies the sex, even of the newly born individuals, has been determined and the two sexes have been treated independently.

At the termination of the period of parturition, as in the sample taken at Durhamville, New York, on June 17, 1931 (fig. 6), there is a conspicuous discontinuity in size of the largest newly born (9.1 mm.) and the smallest individual (14.5 mm.) of the preceding generation. At this period of the year the parent and filial generations are sharply set off one from the other. The curves for the two sexes of the parent generation are so unlike that each must be interpreted separately.

Annandale and Sewell ('21), working on the Indian species *V. bengalensis*, determined by rearing experiments that few males of that species live more than one year, while the females live a normal life span of three years. In the present investigation a strictly statistical approach to the determination of age groups in *V. contectoides* seems to conform to and add support to the observations of Annandale and Sewell on the Indian species. A study of figures 6 to 10 gives evidence that the males of the parent generation, practically all of them, fall between the extremes of 15 and 22 mm., and in their configuration produce a simple population curve. Before the onset of the parturition period in late winter (February 2nd, fig. 3), all of the older generation of males has passed out of the picture and the entire male population is for a time represented by a single group, the members of which are approaching the age of one year. The progressive resolution of the bimodal curve into a unimodal curve as the two modes travel along the axis until the mode of the larger individuals has reached the limit of size set for the species, gives a beautiful demonstration of the limit of the life span of the male in this species. This biological elimination of the older males in late winter offers at least one factor in the explanation of the increased ratio of females to males

(table 1) in that season. According to the foregoing observations, the males live through one breeding season only and die off before they reach the age of two years.

In the course of intensive field observations over a period of more than a year and of incidental observations over a much longer period, the largest male of *V. contectoides* discovered was slightly under 25 mm. in height. From the same habitat that produced this male of maximum size, a female shell 40.7 mm. high was taken. This diversity in size is the resultant of at least two separate factors, relative longevity of the sexes and absolute rate of growth, both of which are apparently internal factors determining the size, beyond the influence or control of environmental conditions. The biological control of growth and biological limitations of life span with differentials for the two sexes seem to be more potent factors in the shaping of life cycles than has been appreciated in the past. This generalization is not limited to the invertebrates, for Van Cleave and Markus ('29) made similar discoveries in working out the life span of the blunt-nosed minnow, except that in this instance the male has a life span longer than the female. Here, likewise, the specific rate of limits of growth seemed to be fixed for each sex rather than for the species as a whole.

In our quantitative samples of *Viviparus contectoides*, the analysis of the life span from the form of the distribution curves is not as simple for the females as for the males. In most of our graphs representing the distribution of the females, at least three fairly distinct groupings are observable when minor irregularities in the curves are ignored. Thus, in figure 6 the mode of the newly born females falls at 7 mm.; another mode stands at 18 mm., representing the mode of the year-old group; the mode at 24 mm. very probably represents the two-year group; while the scattered individuals between 28 and 32 mm. doubtless represent the females in their third year. Tracing this last-named group of large females through the succeeding graphs, it will be noted that in graphs of figures 7 to 10 the representation in this third-

year group becomes progressively smaller. The three-year-old individuals are being eliminated from the population progressively as the new brood assumes its position at the opposite end of the life curve. Later in the summer the modes have shifted slightly and are not as clearly marked as they are at the immediate close of the parturition period. Growth, which had been so rapid in the first few months of free life (figs. 4 and 5), slows down in later life so that other factors which enter preclude a sharp separation of the two- and the three-year groups of females.

Considering the fact that *V. contectoides* attains a greater size than *V. bengalensis*, our data derived from the interpretation of distribution curves checks very closely with the observations of Annandale and Sewell under conditions of controlled experiment, as shown in table 2.

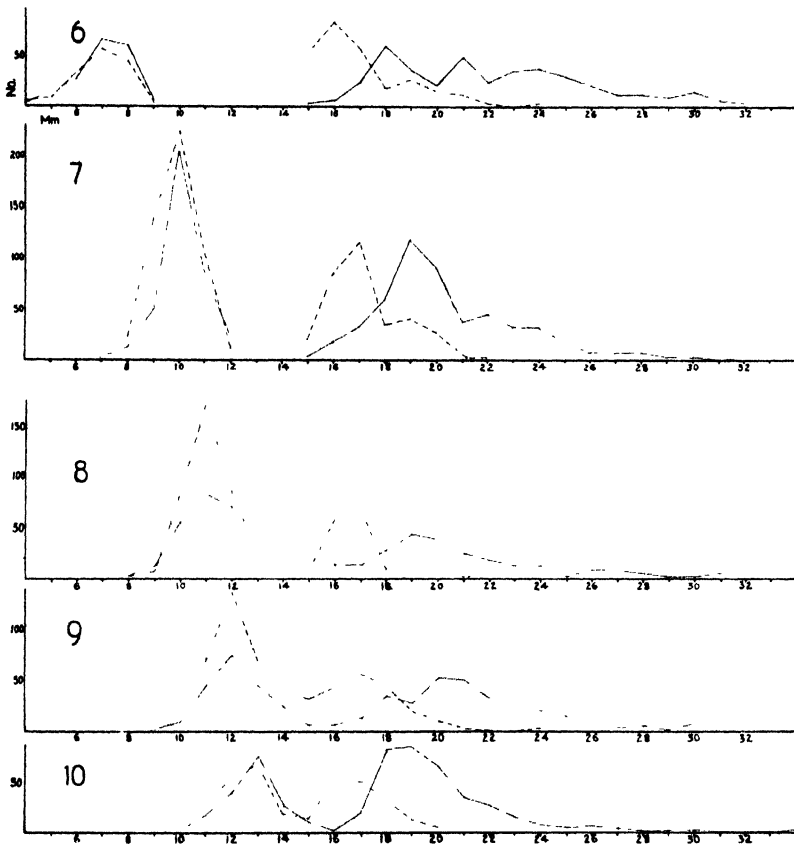
TABLE 2

Comparison in size of age groups of Viviparus bengalensis and V. contectoides, expressed in millimeters of height of shell

	<i>At birth</i>	<i>1 year</i>	<i>2 years</i>	<i>3 years</i>	<i>Maximum</i>
<i>V. begalensis</i> ,	3	15	24	29-30	33
<i>V. contectoides</i> ,	3.8	18	24+	28-32	40.7

Velocity of the initial growth rate

The rapid initial growth rate following the start of free life leaves a broad gap between the largest individuals of the new generation and the smallest of the preceding brood. In a collection taken from the Erie Canal on June 17, 1931, the two yearly groups (fig. 6) are separated by a difference of 6 mm., but at the expiration of less than a month (fig. 7) the gap between the two broods has been reduced to 3 mm. By July 27th, the difference has become still less pronounced until there is a difference of only 2 mm. between the largest of the new brood and the smallest of the preceding one. At the middle of August (fig. 9) the discontinuity between the two curves has been entirely obliterated so that the two originally independent curves have merged to constitute a bimodal or polymodal curve for each sex. The velocity of the growth rate through the first four months, from May to the first of



Figs. 6 to 10 Graphs showing distribution of the sexes and comparisons of height of shell in population samples taken from the Erie Canal at Durhamville, New York, through the summer of 1931. Curves depicting height of male shells in broken line, of female shells in solid line.

Fig. 6 June 17, 1931. Note the conspicuous break in the curves for both sexes, representing size differences between the new brood and the parent generation.

Fig. 7 July 11, 1931. Note reduction in length of break between the young and parent generation.

Fig. 8 July 27, 1931. The mode of the young generation has advanced to 11 mm. Many of the largest females of the parent generation have begun to disappear.

Fig. 9 August 16, 1931. The rapid initial growth rate has completely obliterated the gap between the largest young and the smallest of the parent generation.

Fig. 10 August 31, 1931. The males in late summer conform to a bimodal distribution. All the largest males have died, leaving no male above 20 mm. in height. Relatively few of the three-year-old group of females are present at this time. By midwinter (fig. 3), the males in their size distribution conform to a simple population curve.

September in central New York, is phenomenal. Beyond this point there is a specific check to the growth rate so that many individuals in the second and third years barely maintain themselves and show little or no increase in size. In figure 5, the curves depicting the rate of growth in the second year do not indicate individual increments of growth, but rather the location of the modes for the age groups.

ACCIDENTAL DEATH AND SENESENCE

Aside from the biological limitations to the life span, it would seem that with a snail having the forbiddingly hard shell of a *Viviparus*, the longer it lives the less its chance of meeting with accidental death. Few fishes or other animals which might prey upon the small snails could swallow the two- or three-year-old females of *V. contectoides*. Accidental death is a much more potent factor in the reduction of the population among the smaller individuals than among the fully mature females of this species. Selection of this type is responsible for the progressive diminution in relative numbers of the small individuals of the new generation as shown in figures 7 to 10. At the close of the season of parturition (fig. 7), the mode for the new generation reaches its highest peak, but in each successive graph the relative number of young progressively diminishes until an approximate equilibrium is established in late summer (fig. 10). At this time the young snails seem to have been reduced to the approximate number that is to pass through the winter, a number not much greater than the population of the preceding generation.

There is evidence that at least some of the three-year-old females are sterile (fig. 3), and undergo definite senile changes culminating in death. Under conditions of nature, this elimination of the oldest individuals never results in a wholesale decimation, but is a gradual process. In keeping with this view, only a few of the large dead shells are found at any one time and these occur along with other shells of different ages that have met accidental death.

SUMMARY

1. Periodical quantitative collections of *Viviparus contectoides* from the Illinois River and from the Erie Canal in New York State have been subjected to statistical and biological analysis.

2. More than 3700 individuals have been measured.

3. There is marked dimorphism in the size of the sexes.

4. No shell character is available for determination of the sex.

5. The right tentacle of the male is distinctive of this sex even at birth.

6. No male over 25 mm. has been discovered. The largest female encountered was 40.7 mm. high.

7. The ratio between the sexes is variable, due chiefly to differences in normal life span.

8. Differences in sex ratios may also be attributed to changes in environmental factors leading to segregation of the sexes and to aggregations of the members of one sex.

9. Males live normally but a little more than one year. Females live about three years.

10. In central Illinois, young are liberated from February until May. In central New York, the parturition period seems to begin in March and terminates in June.

11. A gravid female at the same time contains eggs, embryos, and young in the marsupial pouch in a graded series of stages.

12. The smallest marsupial young examined have a shell with one and one-half whorls.

13. At birth the shell consists of three and one-half whorls.

14. Observations of the setae of shells at birth are not in accord with earlier observations. The spiral bands of setae in *V. contectoides* increase from four on the second whorl to six or seven on the last.

15. Color pattern of the shell is laid down and clearly evident on shells at least three months before birth.

16. At birth the shell is approximately 3.8 mm. high.

17. The young shells are disproportionately broad, but growth in height is more rapid than in diameter during the first few months.

18. An extremely rapid initial growth rate slows down to a very slow rate in the second and third years.

19. There is definite correlation between height and diameter of shells of the same age.

20. By the end of the first year most of the shells are 16 to 19 mm. in height. Two-year females are approximately 24 mm. high and those in the third year average about 28 to 32 mm.

21. Graphs are presented showing relative numbers of males and females in populations taken at different seasons.

22. Conclusions regarding life span derived from analysis of statistical data are in close accord with experimental data on other species of the same genus.

23. Accidental death causes a rapid diminution in relative numbers of the newly born individuals during the first three months of life.

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A STUDY OF THE CYTOPLASM IN INSECT OVA

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TWELVE HELIOTYPE PLATES (NINETY FIGURES)

AUTHORS' ABSTRACT

The cytoplasm of the ova of ten species of insects, distributed among the Hemiptera, Coleoptera, Hymenoptera, Diptera, Orthoptera, and Neuroptera, has been studied. The Golgi bodies and chondriosomes were traced. They increase in number by fragmentation, but whether they may also arise *de novo* was not determined. They play no visible part in the formation of yolk or fat or any other structures or substances. Fat and yolk apparently arise independently in the cytoplasm. Vacuoles, which may stain with neutral red, may be present, but they are independent of the Golgi bodies. There is no vacuome in the sense in which Parat and others use the term. The Golgi bodies and chondriosomes are interpreted as substances rather than structures and as intermediate products of metabolism. Other bodies of unknown nature and function are present. The yolk nucleus of *Gelastocoris* is interpreted as a synthetic center.

In this study, I have tried to keep in mind that protoplasm is living matter; that it is constantly changing in the sense that energy transformations are always occurring; that it is a viscous liquid, the viscosity of which is variable; that it is a polyphase colloidal system, and that life is a property of the whole and not of any one part; that the interfaces in this colloidal system are probably the places of energy transformations; that protoplasm exists only in the form of cells, and that the cell is probably the physiological as well as the morphological unit; that protoplasm can neither be divided into living and dead parts nor into visible and invisible structures, except for purposes of description; that it is heterogeneous, yet not without organization; that morphologically, physiologically, chemically, and physically, protoplasm is different in different individuals, and even in different cells of the same individual, yet life is common to all protoplasm; that descriptive terms of either fixed or living protoplasm, such as flar, fibrillar, granular, and alveolar, are words without

¹Contribution no. 229 from the Zoölogical Laboratories, and no. 62 from the Waterman Institute.

fundamental significance; and that one cannot define protoplasm at present except by enumerating properties peculiar to it.

When we reflect that the cell is a unit, it may seem somewhat strange that our cytological studies have concerned themselves largely with first one part of the cell, then the other. It may be the chromatin, the nucleolus, the centriole, the chondriosomes, the Golgi substance, or the cell membrane. Seldom does a paper treat of the cell as a whole. It is probably true that the physiologists, more than the morphologists, have kept this unity in mind. Even the morphologist, however, looks beyond the part or parts with which he works. He has in mind, as does the physiologist, the ultimate understanding of how these parts function and how they are coordinated within the living cell. Even if the present paper does treat of certain limited visible structures within the cytoplasm, and even though I have worked to a large extent with fixed material, I have tried not to lose sight of the cell as a whole. I believe I appreciate fully the limitations of such studies, but I also believe that the study of protoplasm should be approached from all possible angles, and that one group of workers should not become too impatient with the methods of approach of another.

It is with reluctance that I lay aside the microscope, slides, and insects, even for a short time, to write the results of my observations, for it is very certain that I have not extracted the whole of the truth from the cells studied. Changes in the eggs are rapid, and to follow these changes in sections is not easy, even with the aid of living material, for it is difficult to be sure that one has all the stages. On the other hand, correct interpretations are not easy to reach under the most favorable conditions, and the probability is that some or all of my interpretations may be wrong. I make these confessions at the beginning to let the reader know that I believe my observations correct as far as they go, but that I know them to be incomplete; also that I believe my interpretations correct in the light of my observations, yet I know

further study will bring forth new facts and cause modifications of interpretations. The critic may ask why I write the paper. My answer is that if one waited until all questions could be answered with finality, one would never write a paper²

THE PROBLEM

It seems to be generally accepted that chondriosomes are present in some form or other in all animal cells and perhaps in all plant cells. With respect to the Golgi bodies or Golgi substance, the question of universal distribution is not so well established. In fact, some workers, such as Guthrie and Tennent, even doubt the existence of a specific Golgi substance. Other questions concerning both chondriosomes and Golgi bodies, such as behavior, multiplication, distribution, chemistry, function, and whether they are definite persistent bodies or merely chemical substances, remain unanswered for many workers. Bensley, Parat, Dangeard, Guilliermond, and others have failed to convince many with their theory of the vacuome. In fact, all the numerous questions which have been raised during the past thirty and more years concerning the chondriosomes, Golgi bodies, vacuome, and cytoplasmic structures in general remain unanswered. Not only are the questions unsettled, but confusion rather than clarity seems to prevail.

The present paper is an attempt to look at some of these problems and others through a study of the rapidly growing insect egg. The study concerns itself not only with chondriosomes and Golgi bodies, but with all visible changes. As much of the increase in the size of the cell is due to the

² It seems to be a tradition that zoölogists, when writing papers, shall review the literature and enter into endless discussions which in many cases add to the already existing confusion. Such procedures also add to the cost of publication, and shorten the lives of those of us who are compelled to wade through these jungles of words. In writing the present paper, I have made an attempt to forget tradition. Perhaps I have made a mistake, but the experiment seems worth trying once. This does not imply that one can ignore the literature or that an occasional review should not be written.

accumulation of food materials, an excellent opportunity is afforded for a study of the formation of fats and yolk, particularly the part which the chondriosomes, Golgi bodies, nucleoli, etc., may play in their formation.

MATERIALS AND METHODS

The study began with the eggs of *Gelastocoris* and has been extended to include one or more species of Coleoptera, Hymenoptera, Diptera, Orthoptera, and Neuroptera.

Benda, either with or without acetic, and Champy were the two fixatives most generally used. Other methods were tried and abandoned when found less satisfactory. The Benda preparations were in some instances mounted unstained and in others followed by haematoxylin. The Champy preparations were also sometimes mounted unstained or again stained with haematoxylin. Usually, however, they were left either in 1 or 2 per cent osmic acid for five to fifteen days at room temperature or at 35°C. The temperature seems to have no effect, except to hasten the blackening process. Results obtained by fixation were checked by a study of the living cells in direct and dark-field illuminations. Vital stains, particularly neutral red and Janus green, were used extensively.

In spite of the so-called barbarous methods of the cytologist, when the best methods are used and the best results obtained, one is impressed by the remarkable resemblances between fixed and living protoplasm. So close are these resemblances that I think the cytologist has a right to place considerable faith in his methods and his results. He might also ask whether his methods are more apt to give distorted notions of protoplasm than those of the biochemist, when, by complex processes, he extracts substances from the protoplasm, analyzes them, stains them, and leaves one with the general impression that because he is dealing in chemical terms his results are more fundamental and significant than those of the cytologist.

Before proceeding with my descriptions, it may be well to state that I have no absolute criterion for the identification in different species of either chondriosomes or Golgi bodies. I realize clearly that different bodies may be visible following Benda technique, and that structures other than Golgi bodies may blacken with osmic acid. This lack of certainty of identification has led to much confusion in the study of eggs and has strengthened the belief among many zoölogists that such bodies are not definite bodies or structures, but are nothing more than chemical substances which have no more significance than fats or yolk in general. On the other hand, when one works with the male cells of insects during spermatogenesis and sees the constancy of form, appearance, and behavior of chondriosomes and Golgi bodies, one can reach only the conclusion that one is observing the same structures or substances and that they are common to all male insect cells at such times. The criterion for identification here is one of behavior as well as staining reaction. It is also true that in the male cells there are few, if any, other structures with which to confuse the chondriosomes and Golgi bodies. In the insect eggs I have applied the terms 'Golgi body' and 'chondriosome' to structures which, I think, are homologous to the typical Golgi bodies and chondriosomes of insect spermatocytes. Of course, there is greater danger of confusing these bodies with other structures in the egg than in the sperm cells, simply because there are more different kinds of substances in the growing egg and also because their behavior is not well known. While there is some variation in the Golgi bodies and chondriosomes at different times within the same egg cell and in the egg cells of different species, yet there is a remarkable similarity in all cases. So close is this similarity that after prolonged and careful study and even in the face of the difficulties mentioned, I have been forced to the conclusion that these bodies in the several insect eggs studied are homologous structures, and are also homologous to those of insect spermatocytes, and that they can be identified in practically all stages with a high degree of certainty.

GENERAL STATEMENT

When one attempts to digest the literature on cytoplasmic studies, one is amazed at the conflicting observations and theories. Seldom does one find two workers who agree. Neither does it seem possible for any one, however broad-minded he may be, to bring harmony out of this diversity. In fact, the diversity is so great that the task seems hopeless. That many errors in observation have been made is certain, but this is probably inevitable, since there is no way by which the worker can be sure of his identities. Not one of the present-day methods, in my opinion, is infallible. Many different chemical substances have been paraded under the name of Golgi bodies, simply because osmic acid is not specific and will blacken many different substances. No doubt, similar errors have been made with respect to chondriosomes.

Personally, I sometimes wonder whether in this field theory has not outrun established facts. There was a time in the history of zoölogy when facts were interesting in themselves and were allowed to accumulate until sufficiently numerous to point clearly in a given direction. To-day inadequate and unverified observations are woven into theory, with the emphasis on the theory. In making this statement I realize clearly the truth of Whitman's remark when he said that "fact without theory is chaos." He also said, however, with equal emphasis, that "theory without fact is fantasy." Ultimately, of course, we want to know the why of things, but let us not become too impatient with mere facts because they do not reveal to us immediately the chemistry and physiology of cytoplasmic structures. In this study, I have been more concerned with the discovery of verifiable facts than with theory, because, in spite of the hundreds of papers written on this subject since 1898, we need now most of all the plain common verifiable facts which can be had only by prolonged and painstaking observations. Working hypotheses are desirable when they lead to clarity, but not when they lead to confusion.

OBSERVATIONS

Gelastocoris oculatus (toad-bug)

The ovary of *Gelastocoris* is similar to that of other Hemiptera and needs no special description (fig. 1). The oogonia are found at the tip, whence they migrate through the disintegrating food cells and begin their rapid growth shortly before they start their passage down the tube. Food is obtained through the follicle cells and by means of the direct passage of fragments of the food cells through small ducts extending from the eggs to the region of the food cells (fig. 1).

The nuclei of the oogonial cells are relatively large. Within the cytoplasm, following Champy and postosmication, a number of blackened bodies are visible (fig. 2). These bodies may be irregular in shape or in the form of rings or discs, and hence conform somewhat to the Golgi bodies of the male cells of the same species (Payne, '27). At no time have I observed them aggregated near the nucleus. It is possible that some of these bodies are chondriosomes, as prolonged osmication blackens chondriosomes as well as Golgi bodies, but I have no proof that they are, as the ordinary chondriosomal techniques failed to demonstrate them at this time.

After their passage through the food cells, the young oocytes begin to increase in size rapidly and to elongate transversely. In these stages (figs. 4, 5, 6, 9, and 10), two and perhaps three types of bodies blacken with osmic acid, none of which is exactly the same in form and size as those observed in the oogonia. First there are spheres which vary considerably in size and which become black after short or long periods of osmication. Sometimes these spheres show central clear areas following extraction with turpentine (fig. 4), or they may appear gray following longer extraction (fig. 5). A second type is a small granular body (figs. 6 and 9) visible in cells only after prolonged osmication, not observed in figures 4 and 5. These bodies are also present in later stages (figs. 13 and 14), and are interpreted as the

Golgi bodies. The third type, conspicuously observed in figures 4, 5, and 7, is a shorter or longer thick rod, sometimes straight, sometimes bent, and sometimes showing segmentation (fig. 8). This last type of body persists for only a short time, and then is lost completely. It is present in the food cells (fig. 3) as well as the young oocytes, but is rather capricious, as it is not visible in all preparations, nor in all cells of the same preparation. Whether it disappears completely or merely changes its form I cannot say, even though considerable time has been given to its study. These rods may be chondriosomes, but the evidence is not conclusive.

The black spheres, in the early stages of the development of the eggs, have no definite distribution, but as growth proceeds they collect rather loosely in the vicinity of the nucleus, best illustrated in figures 1, 12, and 14. We also find the rods and the small granular bodies collecting in the same region, so that eventually we have a loose aggregation of these three types of bodies. Figure 7 is as late as the rods have been followed, and as this cell lies somewhere between eggs 1 and 2 in figure 1, it is clear that the rods disappear shortly after the eggs start down the tube. The black spheres and the smaller granules remain, however, for some time in close association near the nucleus (fig. 14).

In this figure the aggregate is a somewhat loose one with a central body of some sort occupying the middle region. The nucleus is near the distal end of the egg, using distal to refer to the end of the egg nearest the tip of the ovary. In the stages just preceding, the spheres are arranged in two more or less concentric rings (fig. 12, drawn from living egg). The small granules are distributed apparently at random among and around the spheres (fig. 14).

In the older terminology this aggregate might have been called a yolk nucleus. The term may have been appropriate at one time, but certainly is not appropriate now. After following it through later stages I am inclined to think of the aggregate as a synthetic center—a center where chemical substances are synthesized and move away into other parts of the rapidly growing egg.

Shortly following the stage shown in figure 14, the whole mass of spheres and granules begins to elongate and shows indications of separation into two parts (figs. 1 and 23, showing spheres only). The nucleus meanwhile moves to one side of the cell where it remains (fig. 1). This migration and separation continues until finally there are two distinct parts, one lying at each end of the elongating egg (fig. 1). Usually, and perhaps always, some of the spheres never reach the two separate centers, but migrate to the periphery of the egg where they remain. The small granular bodies which do not reach the centers remain scattered throughout the cytoplasm. As far as the eggs have been studied, the two centers remain in the positions indicated. They are not identical, however, either morphologically or functionally. Before the centers have reached the ends of the egg they are more nearly spherical, especially the proximal one (fig. 31). In a section through the middle region the distal center takes on the general form shown in figures 34 and 35. These sections are cut longitudinally and, of course, the appearance of the section varies with the level of the cut. If reconstructed, they would have the general shape of half or two-thirds spheres with the cut edge of the sphere extended somewhat (fig. 34). The distal center consists primarily of the black spheres and Golgi bodies, although in later stages yolk may be present also. The black spheres move from the center out into the cytoplasm, but may remain near the center for some time (fig. 30). The proximal center is larger and slightly different in shape. The difference can be seen by a comparison of figures 30 and 37 with figures 34 and 35. Oftentimes more or less concentric rings or layers arranged around a fairly large inner mass are visible in the proximal center (fig. 35). It consists of many black spheres and Golgi bodies, but there is more yolk than in the distal center. This yolk is most abundant near the outer edge (fig. 35). The yolk and the black spheres leave the center at this edge and migrate into the cytoplasm. The two centers seem to be made up of essentially the same materials, i.e., black spheres, yolk, and

Golgi bodies, lying in a region slightly darker and more dense than the surrounding cytoplasm. In the region of the proximal center, there are clear indications of streaming movements in the cytoplasm which I am inclined to interpret as the movement of materials from the synthetic centers (fig. 38). The early position of the synthetic center near the nucleus, while formerly thought to be an indication of interchanges taking place between nucleus and cytoplasm, may have no particular significance in *Gelastocoris*, especially since the center, in later development, separates into two parts which move to opposite ends of the egg while the nucleus moves to the side (fig. 1). In these later stages of complete separation of the nucleus and the synthetic centers, the centers continue to persist and to function while the only indication of interchanges between the nucleus and cytoplasm is a zone of clear cytoplasm, usually on one side of the nucleus (fig. 14).

Observations thus far described have been checked in living material unstained and stained in neutral red, and in each case the synthetic centers stand out very clearly. In fact, the outline of the whole is as clear as in fixed material, but the details are lacking. The black staining spheres are clear and hyaline, and the Golgi bodies, if they are visible at all, give the center its golden-brown tint. At least, I know of nothing else which might give this color, and it may be recalled that these bodies in the male cells at the time of acroblast formation exhibit this same tint.

When the eggs are punctured and the contents allowed to flow out, the centers move as units, thus demonstrating that they are more viscous than the surrounding protoplasm.

The experiments with neutral red were not as conclusive as one would like. No results were obtained with eggs after the earliest growth stages. In these early stages, however, penetration is rapid and the results fairly clear. Figures 11 and 12 are two drawings made from living cells stained with neutral red. The shape of the synthetic centers is practically identical with that observed in fixed material and also in unstained living material. A further comparison shows clearly

that the bodies which stain with neutral red are the spheres which blacken with osmic acid, and not what I have interpreted as the Golgi bodies. I have used the term 'black spheres' merely as a descriptive term, because I am not absolutely certain what they are. In many respects they appear and behave as fat, and so I am inclined to interpret them in later stages of development, but in earlier stages they certainly cannot be pure fat. They blacken with small amounts of osmic acid, even with that contained in Benda and Champy fixatives (figs. 30 and 31, fixed in Benda and mounted unstained), and become still blacker with postosmication. Their reaction to turpentine is variable. Sometimes they are dissolved completely, leaving only empty cavities, and this seems always to be the result with prolonged turpentine treatment in the later stages studied (fig. 18). In earlier stages the dissolution may be complete more often than not. Sometimes, instead of vacuoles we may get unstained spheres (figs. 5 and 7), or again the spheres may gradually disappear from view without leaving any traces of spheres or vacuoles (fig. 13). Again the spheres may destain very unevenly in turpentine, giving the appearance of breaking up into small granules similar in size to the Golgi bodies (fig. 36). The facts that the spheres may stain with neutral red and that they are not always dissolved with fat-solvents indicate that they are not fat at that particular time. It would seem logical, in the light of these facts, to interpret these spheres as chemical substances which may at the beginning contain some fatty compounds and which later change into pure fats. Even though they may at one time stain with neutral red, they cannot be interpreted as Golgi bodies. Such observations as these may have led some workers to conclude that Golgi bodies transform into fat.

Because these spheres stain with neutral red and because they transform into fat, they might be interpreted as the vacuome. I think my evidence, however, is conclusive that the Golgi bodies are separate independent structures and that they play no part whatsoever even in the formation of the

spheres. Neither do I think of the spheres, because they stain at one time with neutral red, as a vacuome. In fact, I question the existence of a vacuome in the sense that it is a chemical substance common to all cells and that it can be identified by means of neutral red. If my interpretation is correct, different chemical substances in different insect eggs may stain with neutral red.

It has been stated that in the early oogonial cells there are bodies which blacken with osmic acid, some of which are probably Golgi bodies. It was also stated that in the oocyte, at the beginning of the growth period, there are small granular bodies which I have interpreted as the Golgi bodies. If those bodies present at the beginning of the growth period are the direct descendants of those in the oogonia, it means there has been a change in form. I cannot be absolutely certain, however, that they are directly continuous. From the beginning of the growth period on as far as my studies extended, I think I have been able to follow without loss of identity the bodies which I believe to be the Golgi bodies. At the time of the separation of the single synthetic center into two, and the migration of these two parts to the ends of the cell, the Golgi bodies become in part scattered throughout the cell and in part collected about and within the two synthetic centers, and they remain in these respective positions, except we find in older eggs a greater concentration at the periphery than in the center (fig. 27). As the egg grows, the Golgi bodies also increase in size and may become vesicular (fig. 27).

In the latest stages studied where the yolk occupies most of the space in the egg, all the Golgi bodies are vesicular and at such times are indistinguishable from the vesicular chondriosomes (fig. 26). In still later stages, when the egg becomes filled with yolk, blackened materials may be observed crowded in between the yolk spheres, but all form is lost. The assumption is that this blackened material is both Golgi and chondriosomal material. A definite shape is not a necessity for such materials. The lack of it, however, may have caused

some observers to conclude that there are no Golgi bodies in the mature egg.

Because of the difficulties of technique, I have not been able to demonstrate either the absence or presence of Golgi and chondriosomal substances in eggs approaching maturity.

During the rapid growth of the egg, the Golgi bodies increase in number, and while it is difficult to be certain of their method of increase, the evidence indicates that they multiply by fragmentation. Better evidence of fragmentation is found in other species, however. Golgi bodies are present in the follicle cells as well as in the eggs. Figure 15 shows them ranging from uniformly black masses to vesicles. One often observes in these follicle cells clusters of small granules (fig. 25) giving the appearance of fragmenting Golgi bodies. This type of fragmentation has never been observed in the eggs, but constricted bodies are common.

In the course of these studies, I have not only been concerned with the origin, form, and behavior of Golgi bodies, but also with their function, and accordingly have paid particular attention to their possible relationships to the origin of fat and yolk. Fat seems to arise for the most part in the synthetic centers, but may also arise in the cytoplasm. Yolk may also be present in these centers, but more generally it arises independently as tiny spheres in the peripheral cytoplasm. The Golgi bodies play no part in the synthesis of either yolk or fat.

CHONDRIOSOMES

I have stated that there are some bodies in the oogonial cells which blacken with osmic acid, but that I cannot identify with certainty any of them as chondriosomes. I have also stated that in the young oocytes at the beginning of the growth period there are a number of short thick rods, straight or slightly bent, and which may show segmentation. These rods persist as such for a short time only and then disappear. Whether they fragment or whether the substances of which they are made are transformed into other substances, I cannot

say. If they do fragment the fragments are so nearly like the Golgi bodies that they cannot be distinguished and they do not respond to Benda technique. In eggs considerably later than this, numerous elongated chondriosomal threads appear scattered throughout the cell (fig. 28). Have they suddenly appeared, or have they been present in some other form? There is the possibility and perhaps even the probability that the short thick rods of the early oocytes are chondriosomes, and that these rods fragment into granular chondriosomes which in turn become the elongated chondriosomal threads of the later stages. There is some evidence for this interpretation. First, the rods look like chondriosomes and are invisible with ordinary techniques. Secondly, chondriosomes have been observed to fragment (Lewises on living cells). Thirdly, figure 19 shows a synthetic center in which there are no large black spheres and in which there are many Golgi bodies. In addition to the Golgi bodies there are a few very small threads lying in the cytoplasm around the center. Some of these show a bead-like arrangement. Figure 20 is a portion of this same synthetic center plus the adjacent cytoplasm drawn under a higher magnification. These small threads may be formed from granules and may be the forerunners of the larger chondriosomal threads of older oocytes. This interpretation may not be the correct one, but I have no other which explains the facts quite so well. It would be possible to say that the short thick rods of the early oocytes are micro-organisms and that the chondriosomes of the later oocytes arise *de novo*. While it may be possible for chondriosomes to arise *de novo*, there is not the least evidence for suggesting that the rods are micro-organisms. If the rods are not chondriosomes, a more logical suggestion would be that they are temporary chemical substances different from chondriosomes and Golgi bodies. Other substances, bodies, or symbionts which assume definite shape and form are known to exist in cells (Buchner, '18, and Schrader, '20). In fact, in the eggs of *Gelastocoris*, at a certain stage in their early development there are a number of threads, tubes, or rod-

like bodies in the vicinity of the nucleus (fig. 39). They are not destroyed by ordinary fixatives and stain with acid fuchsin and haematoxylin. They do not blacken with osmic acid. Concerning what they are and their possible function, I have no suggestions, but there is no evidence that they are symbionts.

The chondriosomes, as they appear in figure 28, persist as threads for some time, but by the time the yolk begins to migrate into the center of the oocyte, the threads have begun to change (fig. 27). At this time some of the chondriosomes are still threads; some show swellings; some show the swollen portions vesiculated, but still connected by filaments; while in still others the vesicles have become separated. In a later stage (fig. 26) all chondriosomes are vesicles. It is known that injury to a cell will sometimes cause the chondriosomes to change and even to become vesicular, but these changes in the egg of *Gelastocoris* can hardly be due to fixatives, as they are progressive over a period of time.

In figure 27 there is a narrow belt around the periphery of the egg in which there are many Golgi bodies. They, too, are becoming vesicular, and even at this time one might confuse Golgi and chondriosomal vesicles. In the later stages (fig. 26), one cannot distinguish the two. This is as far as the chondriosomes and Golgi bodies have been followed in drawings. The later stages, where the egg becomes literally filled with yolk, are not workable with the Golgi and chondriosomal techniques. The eggs have been followed sufficiently late, however, to demonstrate that, with the crowding of the yolk and fat-spheres, the Golgi bodies and chondriosomes become wedged in between them and lose their vesicular form. At such times these spheres may be partly or almost wholly surrounded by distorted chondriosomes and Golgi bodies. This arrangement, however, is due wholly to crowding, and not because the chondriosomes and Golgi bodies contribute to yolk and fat formation. In the earlier stages there has been only an occasional contact relationship.

A new type of body or substance, generally ovoid in shape and grayish in color, sometimes exhibiting a black periphery or a central black ring or granule in osmicated preparations, is present near the proximal synthetic center (figs. 29, 32 to 35). They do not stain with ordinary stains, but are visible following all fixatives as grayish bodies, and for want of a better term I shall speak of them as gray bodies. They attain considerable size as they move from the center into the cytoplasm, but are eventually lost. They are never present in the vicinity of the distal center. Whether the center itself is in anyway concerned with their formation, I cannot say, but they have never been observed within the center. In some preparations there seem to be transitional stages between them and the Golgi bodies (figs. 29 and 40), but after prolonged study I do not consider the evidence convincing.

Cicindella sexguttata Fab. (tiger beetle)

The chondriosomes can be dismissed with the statements that they are present in the egg and nurse cells in the form of rods or threads (figs. 58, an ovum; 59 and 60, nurse cells); that in the earliest stages of these cells the distribution seems a random one; that in the early growth period of the egg they are aggregated about the nucleus; that in still later stages they become more uniformly distributed throughout the cytoplasm; and that, so far as they have been studied, they seem in no way connected with synthetic processes nor do they transform into other bodies.

The Golgi bodies are clearer, larger, and more typical of those in the spermatocytes of insects, in general, than those found in the eggs of any other species studied. Because of these facts, I shall describe them and their behavior somewhat in detail. The eggs from the time they begin to increase in size are accompanied by a group of nurse cells (fig. 43), from which the eggs obtain, by direct passage into them through connections made with the nurse cells, part of their food supply. Only the cytoplasm of the nurse cells passes into the eggs. This cytoplasm is very similar to that of the

eggs, that is, there are chondriosomes, Golgi bodies, and some other structures the nature of which is not clear. Whether these transferred structures remain as such within the egg cytoplasm was not determined, but there was some evidence for the disintegration of the Golgi bodies.

At the tip of the ovary the egg and nurse cells are indistinguishable, but soon they become visibly different as the eggs grow more rapidly. At the same time that the size difference makes its appearance, or perhaps earlier, a number of nurse cells collect about each ovum. In the earliest stages studied a small number of typical Golgi platelets are present (fig. 47). There are other materials in the ovum at this time, but, aside from the chondriosomes, they are not clearly differentiated. If we follow the Golgi bodies through the very early growth period, we find that they increase in number and at first are distributed more or less at random through the cytoplasm (figs. 48 and 49). There follows a stage, such as shown in figure 51 and sometimes earlier, when they either migrate to one side of the nucleus or else those in this region multiply more rapidly. At any rate, there is a period when we find many of the Golgi bodies aggregated near one side of the nucleus (shown better in fig. 61). If we study the individual Golgi bodies in these early stages, up to and including figure 51, we find them very much alike in size and appearance. All seem to be platelets. Sometimes the black periphery extends all the way round to form a ring, sometimes only part way round. This difference, however, I do not consider significant. Such typical figures as these lend strong support to the belief that here we have bodies or substances which are homologous or identical with those found in insect spermatocytes. Concerning how this early multiplication of the Golgi bodies takes place I find no evidence. In figure 61, a slightly older egg, the picture is somewhat different. Even among the Golgi bodies clustered near the nucleus there is much variation as to form and size. The picture as a whole gives one the impression that they are fragmenting and migrating out into the cytoplasm. The

peripheral bodies are either uniformly black granules or small vesicular bodies. If one observed these by themselves one might well be skeptical whether they were Golgi bodies, but, with all gradations between them and the large platelets, there can be little question as to their identity. During the stages immediately following that shown in figure 61 there is a marked change in the position of the Golgi bodies. Up to this time most of them were centrally located. Following this stage most of them become peripheral. Figures 54 and 55 are small areas taken from the same egg: 54 from the center of the egg, 55 from the periphery. There is also a marked difference in the appearance of the Golgi bodies in these two figures. Those in the center of the egg are uniform in size, shape, and appearance, while those at the periphery are very irregular in shape and show unmistakable signs of fragmentation. The Golgi bodies still remain peripheral in later stages even after yolk formation begins. I shall return to this stage after discussing some other unknown structures.

In figure 48, an ovum in the early growth period, one observes some typical Golgi bodies and in addition a number of other bodies which are of two distinct kinds. One of them is gray in color, with the periphery slightly darker than the central area. For the most part they are slightly larger than the Golgi bodies, but the two clearly intergrade in size. Sometimes in osmic preparations there is a black granule in the central area. There is a marked resemblance between these bodies and the Golgi bodies, except that the peripheral ring in the Golgi bodies becomes black. One might even imagine that they were Golgi bodies, the periphery of which failed to blacken, or that they were changed Golgi bodies. There is some evidence for both of these points of view. These same bodies are also present in the nurse cells (figs. 59 and 60). In these two figures, however, the peripheral ring has become in most instances as black as the periphery of the Golgi bodies, and furthermore there are all gradations between these bodies and the smaller Golgi bodies. I frankly admit that, for the one who is looking for it, here is sufficient evidence for the

transformation of Golgi bodies into other bodies. I also admit, on the other hand, that here are facts to make even the most ardent orthodox Golgi body enthusiast skeptical about the identity and even the existence of such bodies, in the sense that they are structures which may play a part in heredity, in histogenesis, in secretion, in synthesis of yolk and fat, or in some other imaginary rôle. I say imaginary, because I consider the evidence very scant in support of any one of these suggestions.

Figure 45 is a nurse cell which has reached its maximum size, but before connections with the egg have been established. Here we find a clearer line of separation between the gray bodies and the Golgi bodies. The preparation from which this drawing was made has been destained more than those from which figures 58, 59, and 60 were drawn, and this probably accounts, in part at any rate, for the difference. Some of the gray bodies in figure 45 show two more or less concentric rings. Such a body is also shown in figure 60. We also find in figure 45, as well as in earlier stages, black granules in the center of some of the gray bodies—a fact which indicates the presence of a chemical substance similar to, if not identical with, the peripheral chromophilic rim of the Golgi bodies. Because of the presence of these black granules in the gray bodies and because of the seemingly transitional stages between the gray bodies and the Golgi bodies as exhibited in figures 59 and 60, long hours have been spent in an attempt to find conclusive evidence for some relationship between the two types of bodies. The attempt has been a failure, however, and emphasizes the fact that osmic acid may blacken substances other than Golgi bodies. My first evidence for this conclusion is that these gray bodies are very similar to and perhaps closely related chemically to the gray bodies found in *Gelastocoris*, where no definite relationships between the two were found to exist. In the eggs of other species to be described later, similar gray bodies have been found, and, as in *Gelastocoris* and *Cicindella*, they are independent of the Golgi bodies. In osmic preparations

treated for a longer time in turpentine, as in figure 45, the line of separation between the two types becomes much clearer. Another fact which I consider significant is that the gray bodies are never platelets. They may be spherical, ovoid, or irregular. Some of them are very irregular in figure 45, but this is due to the beginning of changes which result in their disintegration.

The gray bodies which I have just described are very similar to, if not identical with, others which are present about the time that yolk formation begins and which I am convinced arise *de novo* in the cytoplasm. Figures 56 and 57 are two small sections from the periphery of eggs—56 just before yolk formation begins and 57, after many yolk spheres are formed. In figure 56 a peripheral layer of typical Golgi bodies is present. Inside this layer, i.e., toward the center of the egg, are a number of bodies varying somewhat in appearance, among which are a few Golgi bodies. These bodies are in general ovoid in shape. Some of them are gray with a slightly darker thin gray periphery. This kind grades down in size almost to the limit of vision, which facts suggests their *de novo* origin. If the periphery should blacken with osmic acid, the appearance would be identical with that of the Golgi bodies, and I am inclined to believe that in some instances it does blacken. If so, the two bodies would be indistinguishable. The gray bodies, however, as those in the young ovum, never appear as platelets, and so I am inclined to believe the two to be different and to have no relationship to each other.

Other bodies in figure 56 show a thick, dark gray periphery with only a small central region of lighter gray. The central region may, in some instances, be surrounded by a black ring. Are such bodies the same as those with the thinner peripheral ring? I cannot be absolutely certain of this, but in figure 57 the two types clearly intergrade. My tentative conclusion, then, is that the gray bodies observed in figure 48, a young ovum, and those observed in later stages (figs. 56 and 57) are very similar if not identical chemically; that they arise independently of the Golgi bodies; and that they are chemical sub-

stances of a fatty nature which are either stored or used at a later stage in the synthesis of other chemical substances.

As previously stated, there is a third type of body or substance present in young ova (fig. 48). In this figure they are granular with the granules of a slightly darker gray than the ground substance in which they lie, but this appearance is not universal. Sometimes in osmic preparations the entire body is black. If treated with turpentine the black is dissolved, but not uniformly (figs. 49 and 50). Sometimes black granules, or irregular black flecks, or even distinct black rings are clearly visible. If those bodies in which the black rings are visible are studied carefully, clear areas can be seen beneath them. Figure 52B is one of these bodies drawn on a larger scale to show the black rings and clearer areas. If the preparations are treated still longer, the black disappears, but the ring itself remains, now as a dark gray ring. The lighter areas remain about as before. In Benda preparations stained with haematoxylin we find the appearance as in figure 52A. Here the clear areas show as droplets, and the rings are not visible. The general appearance is similar to that of many yolk spheres found in insect eggs, in which there seems to be many smaller fat-spheres, perhaps of a slightly different chemical structure from the substance in which they lie.

I have described these black rings somewhat in detail, because they bear certain resemblances to the Golgi bodies and because of their relationship to the droplets beneath them or perhaps, could we say, within them. My final conclusion, however, is that the black rings are not Golgi bodies. What the structures as wholes are, I cannot say. Their form and behavior are variable. Often in the early stages they are aggregated at one side of the nucleus, the side opposite the Golgi chondriosome aggregate. Sometimes they seem to fuse into a single large body, as shown in figure 51. Eventually they move out into the cytoplasm (fig. 44, a low-power drawing) and disappear.

The eggs of *Cicindella sexguttata* present a beautiful example of what might be termed the vacuome. In figure 61 there are a number of clear droplets lying among the Golgi bodies and chondriosomes, the whole forming an aggregate which is sometimes called the yolk nucleus. The vacuoles, however, as in the cases of the Golgi bodies and chondriosomes, are not confined entirely to this area, but may lie at this time anywhere about the nucleus. In later stages they are found more generally distributed. Since they stain with neutral red in the living cell, they fulfill another specification for the vacuome. As illustrated in figure 63, we find spheres, perhaps of fat, forming within them. It would be difficult to find a prettier picture to demonstrate the formation of secretory droplets, and hence it might seem that the theory of the vacuome is confirmed. I am inclined to interpret the facts differently, however. First, there are Golgi bodies, and I think my figures leave no doubt on this point, which are entirely independent of the vacuoles. In a recent paper, Nath ('31), contrary to his earlier statements, has reached a similar conclusion. Secondly, there is no indication of the formation of the vacuoles by the Golgi bodies. Thirdly, these vacuoles which stain with neutral red are not the same as those which stain with neutral red in *Gelastocoris*. Hence, in my opinion, neutral red is no safer than osmic acid, perhaps not as much so, as a method of identification of the same chemical substances. If this be true, then there is no common chemical substance distributed in the form of vacuoles, canals, or otherwise, which can be designated as the vacuome.

Cicindella repanda Dej. (tiger beetle)

Figure 62 is a section of the egg of *Cicindella repanda*. In this cell it is interesting to note that the vacuoles are for the most part at one end of the cell rather than near the nucleus, and there are no more Golgi bodies and chondriosomes here than elsewhere. Such facts as these lead me to question whether the aggregation of vacuoles, Golgi bodies, and chondriosomes near the nucleus as in figure 61 has any

particular significance. This does not mean that I question the interchange of chemical substances between nucleus and cytoplasm. In fact, in this same drawing we find a region of granular cytoplasm free from inclusions, which certainly is a clear indication of activity of some sort which is different from that going on in the rest of the cell. But this is not conclusive evidence that the nucleus is concerned in it, for we find a similar region of clear cytoplasm at the end of the same cell.

Colletes inaequalis Say (bee)

Primarily, because gray bodies are found similar to those described in figures 56 and 57 of *Cicindella sexguttata*, a few figures of one of the solitary bees are included. Figure 65 is a small section of the proximal end of the egg (X in fig. 64). These bodies are formed in this region in large numbers before they appear elsewhere. Since there are no more Golgi bodies in this region than elsewhere, and since there is no visible relationship between the two types of bodies, I can reach only one conclusion, and that is that they are separate, independent entities. Personally, I must admit that if these gray bodies were interspersed with the smaller gray bodies described in figures 56 and 57 (*Cicindella*), I could not distinguish one from the other. Since there seems little doubt of their independent origin in the bee, my conclusion that the gray bodies in *Cicindella* arise de novo is strengthened. In figure 66, a section from the periphery of an older egg, we find a beautiful example of yolk or fat-spheres forming within larger, more liquid droplets.

Tropisternus glaber Hbst. (beetle)

In the young ova of this species we find a method of formation of fatty material slightly different from, but still independent of, the Golgi bodies and chondriosomes. Figures 67 to 72, drawings from Champy and osmic preparations, represent a series of stages which tell the story clearly. In figure 67, an early oogonial cell, are a number of black bodies which

I have interpreted as the Golgi bodies. Nothing else is visible. In figure 68, as the egg begins to increase in size we find irregular, stellate, or amoeboid-like masses which certainly show no relationships with the Golgi bodies. Figure 69 shows the same thing, only the masses are more numerous. In figure 70 most of them have moved to the periphery of the egg, while in figure 71 they are clearly separating into a number of small spheres. In a still later stage (fig. 72) the separation is complete, and we see nothing but clusters of small spheres.

I have spoken of these spheres and the irregular masses from which they arise as fatty material. They are not true fats, however, because they do not blacken in osmic nor do they stain with haematoxylin. The color within the fixed cell is gray or slightly brownish.

It should also be noted that the Golgi bodies are for the most part rod-like rather than typical platelets and that the rods exhibit no chromophobic material.

Anisolabis annulipes? (earwig)

A few sections of the eggs of *Anisolabis* are included primarily because, in the young ova, I found gray bodies very similar to those described in *Cicindella*, *Colletes*, and *Gelastocoris* (fig. 74). As in the other species, they do not blacken readily with osmic acid except occasionally a central granule or an inner ring. Neither do they stain with haematoxylin. They are generally ovoid in shape, and the size varies by small gradations from the largest to the smallest. The large black spheres are fat. In the same figure are a few small black rings, which are Golgi bodies. They are better shown in figure 75, a section of an egg drawn on a larger scale than figure 74. The chondriosomes stand out very clearly in the form of rods. Figure 76 shows a few individual Golgi bodies, showing variability as to the size of the body and also some constrictions indicating clearly that the bodies divide. In no case have I observed any relationships between the chondriosomes, Golgi bodies, and other visible structures

within the cytoplasm. Yolk, fat, and gray bodies are independent in origin.

Tabanus atratus Fab. (fly)

Figure 80 is a section of a young egg, and is included because it demonstrates the presence of Golgi bodies and gray bodies which again are similar to those previously described. Note the presence of the central black granule in some and also that the peripheral ring may blacken. The Golgi bodies at this time are uniformly black. Here, as in previous cases, one may imagine transitional stages between the Golgi bodies and the gray bodies, but I am inclined to interpret them as separate unrelated structures.

Dineutes assimilis Aubé (beetle)

Figures 77, 78, and 79 are three successive stages in the early development of the eggs of this species. The Golgi bodies in figure 77 are uniformly black and irregular in shape, but in figure 78 and particularly in 79 they are in part vesicular. The changes are clearly progressive. The unusual thing in this egg is the presence of two large gray spheres in the very young ovum (fig. 77). They are not the same as those gray spheres described in the other species as they are uniformly gray. Just what they are chemically I do not know. As they do not blacken with osmic acid they cannot be true fats. Neither are they yolk spheres in the ordinary use of this term. They increase in number, as illustrated in figures 78 and 79. My interpretation is that they are chemical substances formed at this time to be used in later stages of growth. They are independent of the Golgi bodies.

Oecanthus quadripunctatus Beut. (cricket)

The early stages of this species have been studied in some detail because of some marked changes which occur in the Golgi bodies. In the oogonial cells before growth begins (fig. 87), the facts are difficult to obtain. We find in the figure drawn a mass of material with an outer dark periphery.

It may be made up of Golgi substance only, or it may include chondriosomal substance as well. One cannot be sure what it is. In the early growth period (fig. 81) the mass has disappeared, and we find a number of separate irregular black bodies (osmic preparations), two of which are distinctly bipartite with a gray chromophobic substance between the two parts. Whether these separate bodies have arisen by the fragmentation of the original mass, one cannot be certain, but the inference is that they have so arisen. As growth proceeds, the number of black bodies, or Golgi bodies, as I now interpret them, increases (fig. 82). For the most part, at this stage, the Golgi bodies are uniformly black, but a few are becoming vesicular. At a still later stage (fig. 83, the distal end of the egg) the number of vesicles has increased considerably. The distal end of the egg was drawn, because the vesicles form here in large numbers before they appear elsewhere in the egg. They are clearly ovoid in shape, with a distinct dark periphery, and the longer sides of the ovoid body are often much thicker than the shorter sides. Sometimes the inner part of the vesicles blackens with osmic as well as the periphery, but in destaining the central part gives up the stain first. The vesicles at this time do not appear to be platelets, and I am inclined to believe that some of them never become such. In figure 84, again the distal end of the egg, but a later stage in development, there is a larger number of vesicles, the appearance of which is similar to those in figure 83, except a few do appear to be platelets and others show constrictions indicative of division. Not all the rods observed in this figure, however, are platelets looked at from the edge. Some of them are formed directly from the original Golgi bodies without passing through the vesicular stage. The exact fate of the vesicles was not determined with certainty, but what evidence I have indicates that they disappear during early yolk and fat formation, and that they do not transform directly into fat or yolk, nor do fat and yolk spheres form within them as secretion products. Figure 85 is a section from the periphery of the egg shortly after yolk for-

mation has begun. The gray spheres are yolk and the clear droplets are filled with a watery substance, in which at a later stage (fig. 86) fatty substances are deposited. Only a few of the Golgi bodies at this time and in figure 86 are vesicular. For the most part they are straight or slightly bent rods, but may appear also as irregular bodies. These observations lead me to conclude that in this species the Golgi bodies may become large vesicles and that such vesicles disintegrate, giving rise perhaps to released chemical substances to be used in other synthetic processes. They play no direct part in the formation of fat and yolk.

Oecanthus nigricornis Walk. (tree cricket)

Three figures (88, 89, and 90) of this species are included to show certain resemblances to *quadripunctatus* and also certain differences. In figure 88 is a cluster of vesicular bodies which separate as in 89. I interpret these as the Golgi bodies. In figure 88 there is also present a large gray body with a slightly darker periphery. Whether it is similar to the gray bodies which I have described in other species, I cannot say, for it is lost in the following stages. In figure 90 the number of Golgi bodies has increased, and some of them have become vesicular similar to the vesicles in *quadripunctatus*, but different in that the vesicles become larger and blacken more uniformly with osmic. Preliminary experiments indicate that these vesicles stain with neutral red.

Stone-fly, sp.?

Two figures are included primarily because, in all other species studied, yolk and fat arise at the periphery and migrate centrally, while in this stone-fly yolk and fat are at first located around the nucleus and migrate peripherally (fig. 41). Outside this layer of yolk, fat, and Golgi bodies is a layer of dark granular material, which in later stages (fig. 42) breaks up into irregular masses. Because of insufficient material the fate of these masses was not determined with certainty, but there were some indications that they separated into small spheres, perhaps of a fatty nature.

SUMMARY AND CONCLUSIONS

As stated in the beginning, I shall not discuss the literature at length, primarily because I see no way as yet of bringing order out of the great diversity of published statements and opinions concerning visible structures or substance in the cytoplasm. I say this, even though Bowen, in a series of excellent papers, has attempted to do so. Everywhere one meets not only the difficulty of interpreting the facts, but also the difficulty of knowing what the facts are. In reading the literature, one feels certain that many mistakes in observation as well as interpretation have been made, but to correct the errors from the published figures and statements is hazardous, to say the least. The recent work of Beams in reexamining the same material on which others have worked demonstrates clearly that a large number of errors have been made. I have about reached the conclusion that I have no right to criticize the facts of another worker unless I have carefully studied the material myself, and even then I might be skeptical as it is often difficult or impossible to get the facts.

The present study has been limited to the eggs of ten species of insects in five different orders, and my conclusions will be limited to the same material. In the light of past work, notably that of Gatenby and Nath, one might expect to find conflicting evidence in these different species, but such has not been the case. Each supports the other in contributing to the general conclusions reached.

Whether we wish or not, we must candidly admit that there is no absolute criterion for the identification of Golgi bodies or a Golgi substance, if the latter term is preferable. The osmic methods are more generally used than others, and certainly no one can say that osmic is specific. Neutral red, to my point of view, may stain different chemical substances. Furthermore, the vacuoles and the Golgi bodies are independent substances. Other methods are equally capricious. If these statements are true, and I think they are, it seems certain that many different chemical substances have been

paraded under the name of Golgi bodies, or Golgi apparatus, or Golgi material, or Golgi substance (take your choice). Some workers, such as Guthrie and Tennent, even doubt the existence of such a chemical substance common to all cells. While I am inclined to agree with Guthrie and Tennent that we are dealing with a substance rather than with structures, my evidence in the insect eggs will not permit me at this time to deny the existence in these cells of a common chemical substance which may be called the Golgi substance. This statement does not imply, however, that the substance is identical chemically at all times in the same cell or in different cells. Neither does it imply that I know what this chemical substance is. Perhaps it is a lipin in combination with a protein, but, as Tennent has pointed out, this means little when one has said it. While I admit frankly the difficulties involved in the identification of the Golgi substance, I believe that in most stages studied, it can be identified with a high degree of certainty, by checking with different fixatives and stains, by a study of living material, and by following its behavior. I also believe that the Golgi substance of the insect egg is the same as that described in the male germ cells of insects.

The possibility that the Golgi bodies may be independent self-propagating structures has led to various theoretical discussions similar to those indulged in by students of chondriosomes. The suggestions that they may be factors in such vital processes as fertilization, heredity, differentiation, and localization may be dismissed with the statement that such suggestions are highly speculative and that there is little or no evidence in their support. The possibilities that the Golgi bodies are secretory in function, and that they may play an active rôle in yolk and fat formation deserve more serious consideration, for here there are some supporting facts.

As the present paper is limited to a study of the growth of insect eggs, I shall not discuss the secretory hypothesis in its general aspects. In recent years a number of papers dealing with fat and yolk formation have appeared. Some of these workers, Gatenby, Woodger, Harvey, Weiner, and

Steopoe, ascribe yolk formation to the Golgi bodies; others, Hirschler, Gatenby, Brambell, Ludford, Nath, and King, describe the formation of fat either directly or indirectly from the Golgi bodies. In reading these papers one finds a wide divergence of opinions, and apparently in facts. The same author (examples: Gatenby and Nath) may describe fat formation by different methods in different species.

In the insect eggs studied, only a few Golgi bodies are present in the oogonial cells, but as the eggs grow in size, the Golgi bodies increase in number rapidly. This is particularly noticeable in *Oecanthus*. As yolk and fat formations progress, they seem to decrease in number, at least relative to the amount of cytoplasm present. In later stages, especially as observed in *Gelastocoris*, they lose their identities as bodies and become wedged in between the yolk and fat spheres. Whether all the Golgi substance is used during growth, and hence absent in the mature egg, as maintained by Tennent, could not be determined. As far as my observations go, I have found no evidence which would lead me to conclude that the Golgi bodies are concerned either directly or indirectly in either yolk or fat formation. Sometimes Golgi bodies, chondriosomes, and yolk and fat spheres are aggregated near the nucleus to form what has been termed the yolk nucleus. This aggregate and its position near the nucleus has led to many suggestions, among which is the one that the Golgi bodies are concerned with synthetic processes. To me such associations do not furnish evidence. At best they can be suggestive only. Such an aggregation is certainly a center of activity, for the Golgi bodies and chondriosomes increase in number at this time. Even fat-spheres and other droplets of various sorts may arise therein, but I have failed to find any direct connection between these new formations and the Golgi bodies. Neither does contact between Golgi bodies and yolk and fat-spheres supply convincing evidence that the Golgi bodies contribute to their formation. As the cell becomes crowded with fat and yolk, such contacts are inevitable.

The Golgi substance is present in insect eggs in the form of separate, distinct granules or masses which are commonly called the Golgi bodies. These bodies may change in size and form during the growth of the egg of a given species and are variable from species to species. Sometimes they appear as irregular, uniformly blackened bodies in osmic preparations, or they may be vesicular and plate-like with a chromophilic periphery and a central chromophobic region. The black periphery may or may not extend all the way round the vesicle. Hence they have no characteristic form, and my conclusion would agree with Bowen that it is the substance which is essential, although I would not agree with him as to function.

If there is no Golgi substance, then there is no question about its origin and increase in amount. Granting, however, that there is such a substance, its origin and multiplication become questions of interest. In the insect spermatocytes the Golgi bodies, or the dictyosomes of some authors, seem to increase in number by fragmentation. At least this is the conclusion of Bowen and others. Whether they always arise in this way remains questionable. In the insect eggs there are unmistakable signs of constriction and fragmentation, particularly in *Gelastocoris* and *Cicindella*. Whether they may also arise *de novo* could not be definitely determined. If the Golgi substance does fragment or divide, the fact is somewhat difficult to harmonize with the view that the Golgi substance is an intermediate product of metabolism. Other products, such as fats and yolk, do not divide.

In some instances, particularly as described in insect spermatocytes by many authors, the Golgi bodies exhibit a differentiation into two parts, commonly known as the chromophilic and chromophobic portions. Apparently the same individual body, in the insect eggs at any rate, may change from a condition where it is wholly chromophilic to one where the two parts are clearly visible. Such facts lead to the question whether the chromophobic portion is always a constituent part of the body, or whether it is a differentiated

product of the chromophilic portion. As I have previously stated, I do not consider the chromophilic periphery an active chondriosome. Neither do I think of the chromophobic portion as a secretion product of the chromophilic part any more than I think of oxychromatin as a secretion product of the basichromatin. I prefer to think of the two parts as a result of differentiation, and attach no further significance to it.

In the insect eggs the Golgi bodies have never been observed to give rise to granules or droplets similar to the proacrosomic granules.

The presence of Golgi bodies within cells at all times, if such a fact is eventually established, can be explained by assuming that the presence of certain chemical substances is necessary for the life of a cell. That they may be intermediate products of metabolism, as assumed by Guthrie and Tennent, seems logical.

Whether the Golgi substance is present in the form of vacuoles such as have been described in the invertebrates is questioned by Bensley, Dangeard, Guilliermond, Parat, and others. According to these workers, the typical Golgi platelet with its chromophilic rim is an artifact. The essential thing is a series of watery droplets, the vacuoles, or collectively the vacuome. They also maintain that a dilute solution of neutral red when applied to the living cell is specific for these vacuoles.

My observations show clearly the presence of both vacuoles and Golgi bodies in *Gelastocoris* and *Cicindella*, and while I have attempted to discover some relationship between the two types of structures, no such relationship has been found. In all my observations the vacuoles and Golgi bodies are separate independent structures—a conclusion formerly reached by Gatenby, Nath, and Beams. Neither have I seen any indications of small secretory droplets arising within the Golgi bodies, which later grow into the vacuoles. In both *Gelastocoris* and *Cicindella* the vacuoles stain with neutral red, yet the behavior and fate of the two are different. In *Gelastocoris* they blacken with osmic acid from the begin-

ning and seem clearly to change into fat. In *Cicindella* they never blacken with osmic, and instead of changing into fat, yolk-like spheres accumulate within them. Certainly, the vacuoles in these two species cannot be alike chemically; yet both stain with neutral red. From the evidence in the insect eggs, then, there is no vacuome in the sense in which this term has been used, that is, in the sense of a given substance common to all cells.

There may be fairly general agreement as to the identity and distribution of chondriosomes, but there is not agreement as to their origin, chemistry, and physiology, and as a consequence many different functions have been assigned them. The thesis that they are independent, self-propagating units is essential for most of these theories, although such a thesis is by no means established. Benda, Meves, and others (based upon observations of the behavior of chondriosomes in maturation and fertilization) have suggested that they may play a part in heredity. Kingsbury, supported by others, has developed the theory that they are concerned with respiratory functions. Regaud thought of them as centers of chemical action, and variations of this idea are found in the works of Loyez, Hirschler, Brambell, Gatenby, Hogben, and King, who attribute yolk formation either directly or indirectly to the chondriosomes. Others have traced glandular secretions, pigment formation, etc., to them. Benda, Meves, and Duesberg, in particular, have traced the transformation of chondriosomes into various fibrillar structures, such as myofibrils and neurofibrils, and Levitsky, Pensa, Guilliermond, and Meves have described the origin of plant plastids from them. In fact, they have been accused by one author or another of producing either legitimately or illegitimately about every visible substance in the cell.

That the granules within the cytoplasm may be elementary microorganisms was suggested by Altmann. More recently Portier and Wallin have attempted to demonstrate that chondriosomes are, in fact, bacteria which have become adjusted to an intracellular existence.

Finally, some workers, among them Guthrie and Tennent, think of the chondriosomes as a chemical substance, which in turn is an intermediate product of metabolism.

Without discussing these suggestions, I wish to state that there are no indications in the insect eggs studied of the transformation of chondriosomes into fats, yolk, fibers, or any other substances or structures. Neither is there any evidence of enzymatic or catalytic activity. The facts, as I have seen them, favor the interpretation of Guthrie and Tennent in so far as function is concerned.

Even though the periphery of the Golgi bodies may blacken with osmic acid, and even though it may stain with Janus green, I cannot interpret such a periphery as an active chondriosome. In fact, I see no reason for attempting to separate chondriosomes into active and inactive groups.

In *Gelastocoris* we find first one and later two aggregates of Golgi bodies, chondriosomes, vacuoles, fatty substances, and other unknown substances, which may in older terminology be called yolk nuclei. Wilson ('25) regards the yolk-nucleus complex, even though the term has been applied to diverse groups of substances, as the homologue of the idiozome of the spermatocytes. It is true that the two do bear certain resemblances when within the vicinity of the nucleus, but, when the yolk nucleus separates into two parts which move to opposite ends of the egg as in *Gelastocoris*, homologies become questionable. I have interpreted the yolk nucleus as a synthetic center, a center of great activity where yolk, fat, vacuoles, and other chemical substances are synthesized, and question whether its early position near the nucleus has any particular significance.

While I do not mean to deny nuclear activities, and their effects upon the cytoplasm, the nucleus seems to play no rôle in the synthesis of the enormous accumulations of yolk and fats during the growth of insect eggs.

Gray bodies, similar in appearance and perhaps similar in chemical composition, are present in the growth period of most of the eggs studied. Apparently they arise *de novo*,

grow in size, and are eventually lost. Their rôle is probably a metabolic one, similar to that of fats in general.

In the eggs of *Gelastocoris*, shortly before the nucleus moves to the side of the cell, there are eight to ten elongated rods or tube-like bodies in the vicinity of the nucleus. They remind one somewhat of the canals described by Schröder ('20) in the white fly, *Trialeurodes vaporariorum*. There is no evidence as to their origin, behavior, fate, or chemical composition.

Hogben, Gatenby, Ludford, and Nath have described yolk formation from nucleolar extrusions. In my own studies, however, I have seen no evidence of such extrusions. Since yolk and fats have no relationships to other visible substances in the cell, the assumption is that they arise independently in the cytoplasm.

In the older literature on the physiology of the cell, we often read that the nucleus lies at the center of activity. I refer particularly to such observations as those of Haberlandt on plant cells and Korschelt on egg and nurse cells of insects. I do not intend to discuss this literature, but do wish to say that, in the insects studied, the nuclei play no visible parts in the synthesis of fats and yolk. Neither do they lie at the source of food supply. The only indications of nuclear activity are the synthetic center and a clear zone of cytoplasm near the nucleus in the early stages of the growth of the egg. Korschelt and others have been inclined to interpret the amoeboid shape of the nucleus in the nurse cells as evidence of great activity, perhaps in the synthesis of foods for the egg. Personally, I question this interpretation, particularly as greater activities, both as to synthesis of foods and increase in the ground cytoplasm, are going on in the egg cells than in the nurse cells. I have never observed an egg nucleus with such amoeboid processes as Korschelt has described in *Vanessa*.

It is fortunate that a comparative study of several different insect orders has been made, because I find the facts essentially the same in all. This, it seems to me, is particularly

significant, especially since Gatenby and Nath have described marked differences in methods of yolk and fat formation in different species.

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PLATES

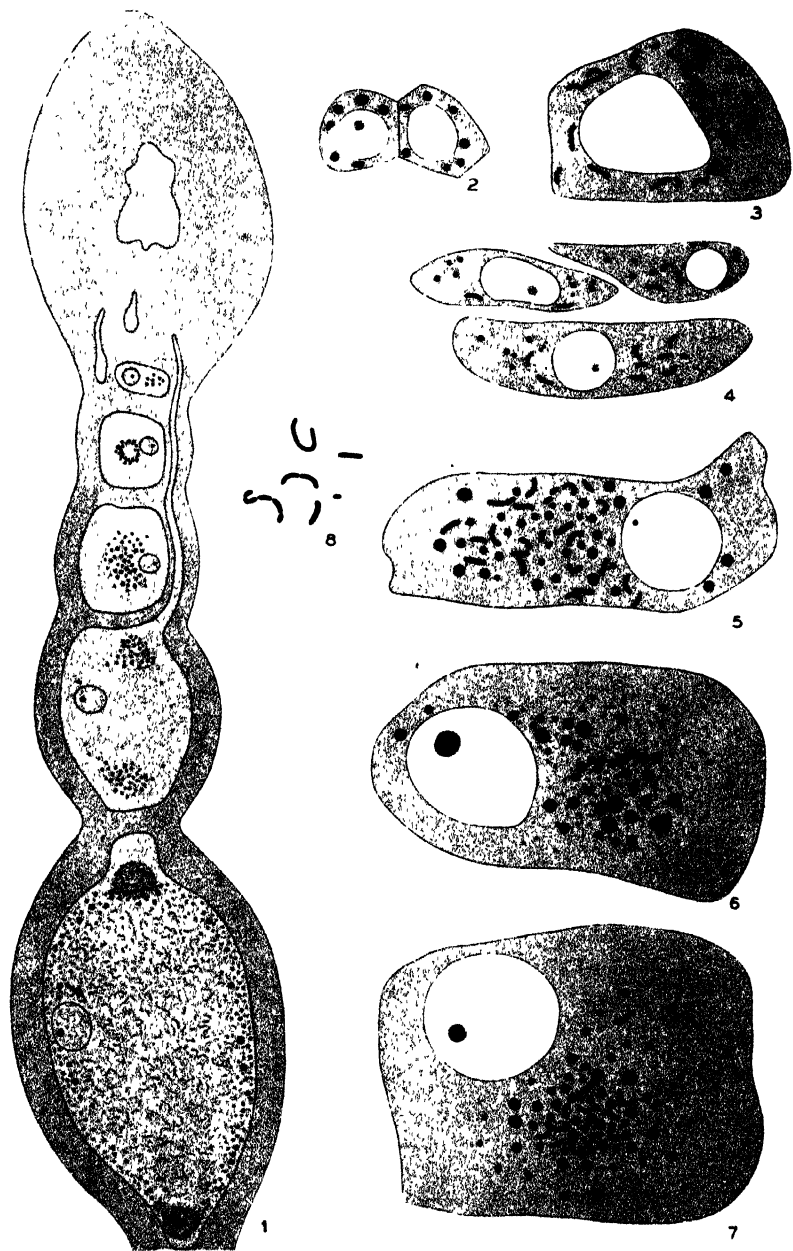


PLATE 1

EXPLANATION OF FIGURES

All figures of *Gelastocoris oculatus* fixed in Champy, followed by osmic and turpentine, drawn to same scale. Details of food and follicle cells not given.

1 An ovarian strand, showing eggs in early growth period. Note position of nucleus and yolk nuclei.

2 Oogonial cells with black bodies, presumably Golgi bodies.

3 A food cell from tip of strand, showing numerous rods which may be chondriosomes.

4 Three ova at beginning of growth period. Rods are present and also some small spheres.

5 Young ovum with rods and unstained spheres.

6 Young ovum about same stage as 5. Note absence of rods. Spheres are black. Small black granules are interpreted as the Golgi bodies. Not destained as much as figure 5.

7 Shows same structures as figure 5, but at a later stage. This is as late as rods have been traced. Unstained spheres are same as black spheres in figure 6.

8 Some of the rods drawn individually to show variation in size, and also to show segmentation.

PLATE 2

EXPLANATION OF FIGURES

All figures of *Gelastocoris oculatus*; 11 and 12 from living cells stained with neutral red the rest fixed in Champy followed by osmic and turpentine. ;

9 and 10 Young ova, showing the granular Golgi bodies and large black spheres.

11 and 12 Young ova stained with neutral red. The spheres in synthetic center stain pink. These same spheres are hyaline droplets in unstained living cells and black in osmicated material.

13 Ovum showing Golgi bodies. The turpentine has caused disappearance of spheres. Rods are no longer present.

14 Ovum somewhat later than figure 13, but drawn on a smaller scale. Shows synthetic center consisting of Golgi bodies and black spheres. Note region of clear cytoplasm in contact with nucleus.

15 Follicle cells, showing large vesicular Golgi bodies and chondriosomes massed near ends of cells in contact with egg.

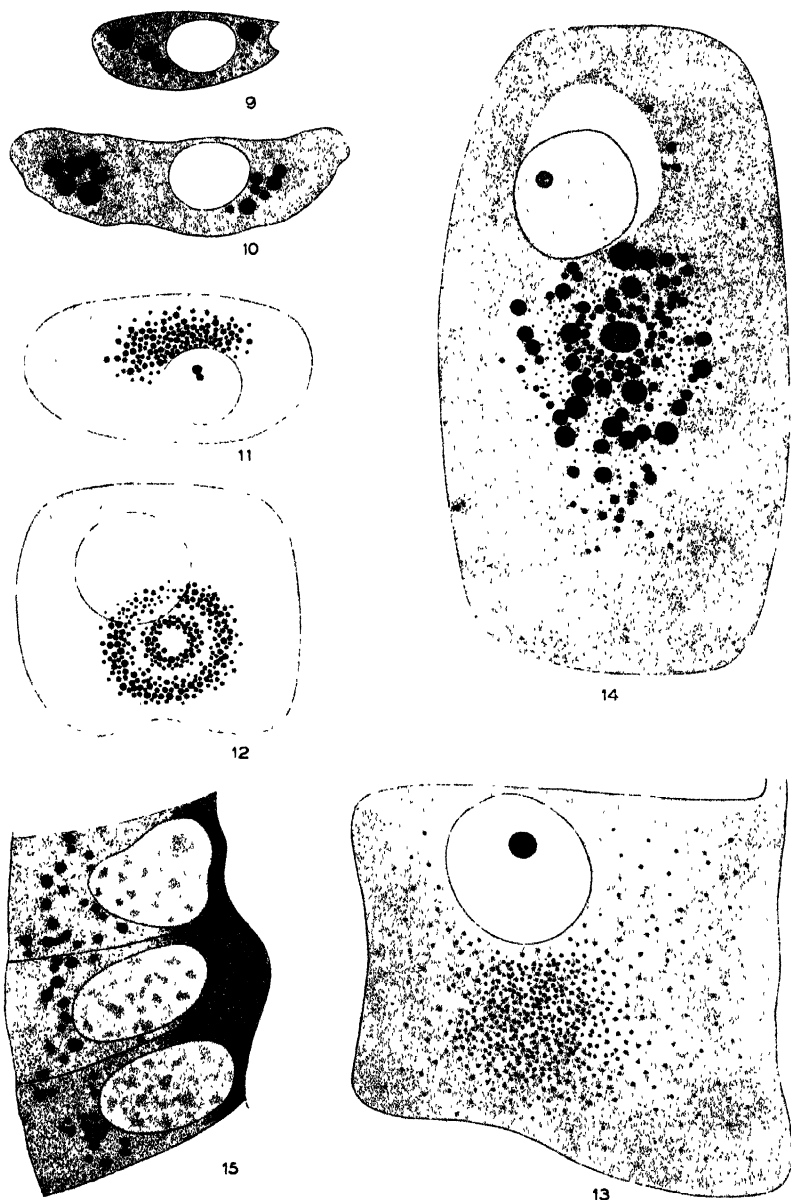


PLATE 3

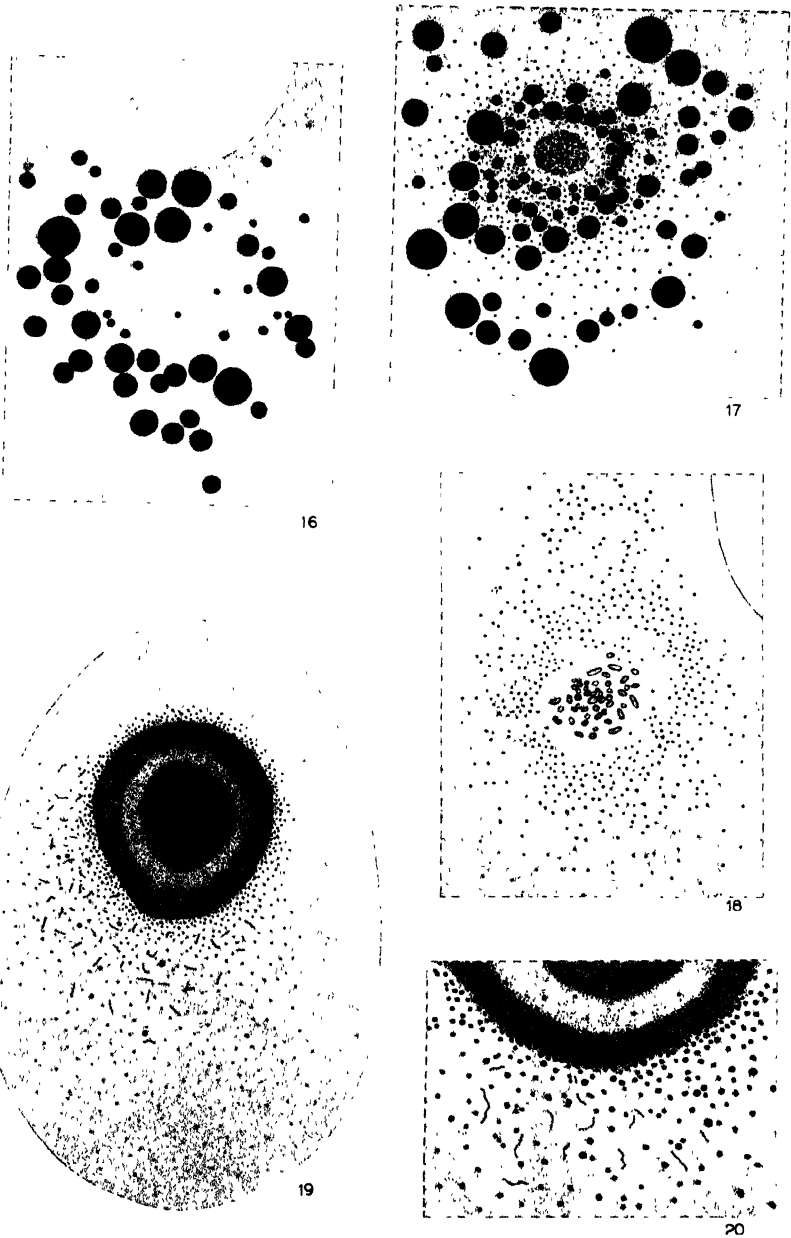
EXPLANATION OF FIGURES

All figures of *Gelastocoris oculatus*; fixed in Champy and all except 16 followed by osmic and turpentine.

16, 17, and 18 Synthetic centers in eggs in about the same stage of development. Figure 16 shows the center following Champy fixation, but unstained and not osmicated. The black spheres only show. Figure 17 fixed in Champy plus osmication. In addition to the black spheres are the small granular Golgi bodies. Figure 18 is the same as 17, except treated with turpentine. Black spheres have been dissolved leaving vacuoles.

19 Ovum with synthetic center. Note the small threads.

20 Part of same synthetic center shown in figure 19, drawn on a larger scale. Golgi bodies are becoming vesicular. The small threads are interpreted as chondriosomes.



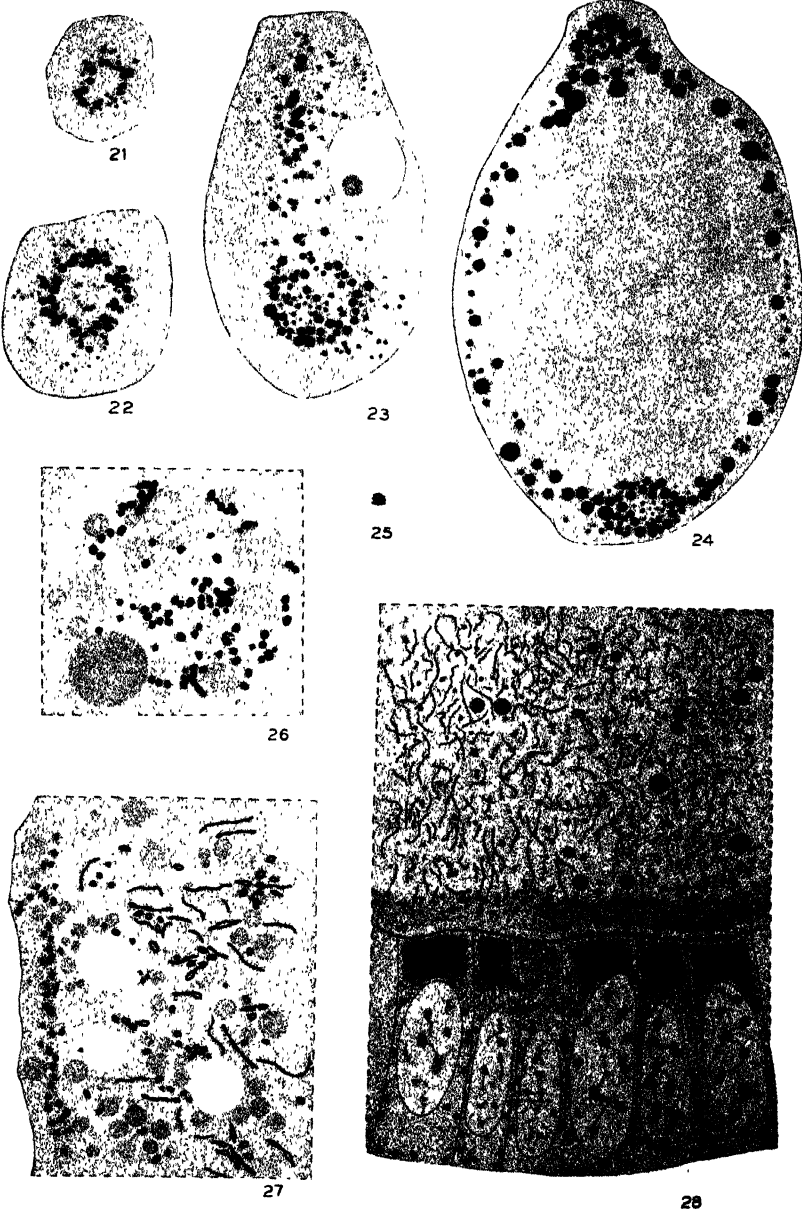


PLATE 4

EXPLANATION OF FIGURES

All figures of *Gelastocoris oculatus*.

21, 22, 23, and 24 Champy fixation, unstained. Four eggs in a row from one egg strand. Note migration and distribution of black spheres with the separation of the synthetic centers.

25 Possibly a fragmenting Golgi body in a follicle cell.

26 Central region of ovum, showing yolk spheres and vesicular Golgi bodies and chondriosomes. The two are indistinguishable. Champy plus osmic.

27 A small section of the periphery of an egg, showing vesicular Golgi bodies at periphery, and chondriosomes which are breaking up and becoming vesicular. Yolk spheres and dissolved fatty vacuoles are also present. Champy plus osmic plus turpentine.

28 A small section of the periphery of an egg with the follicle cells. The threads are chondriosomes and the black granules are the Golgi bodies. Champy plus osmic plus turpentine.

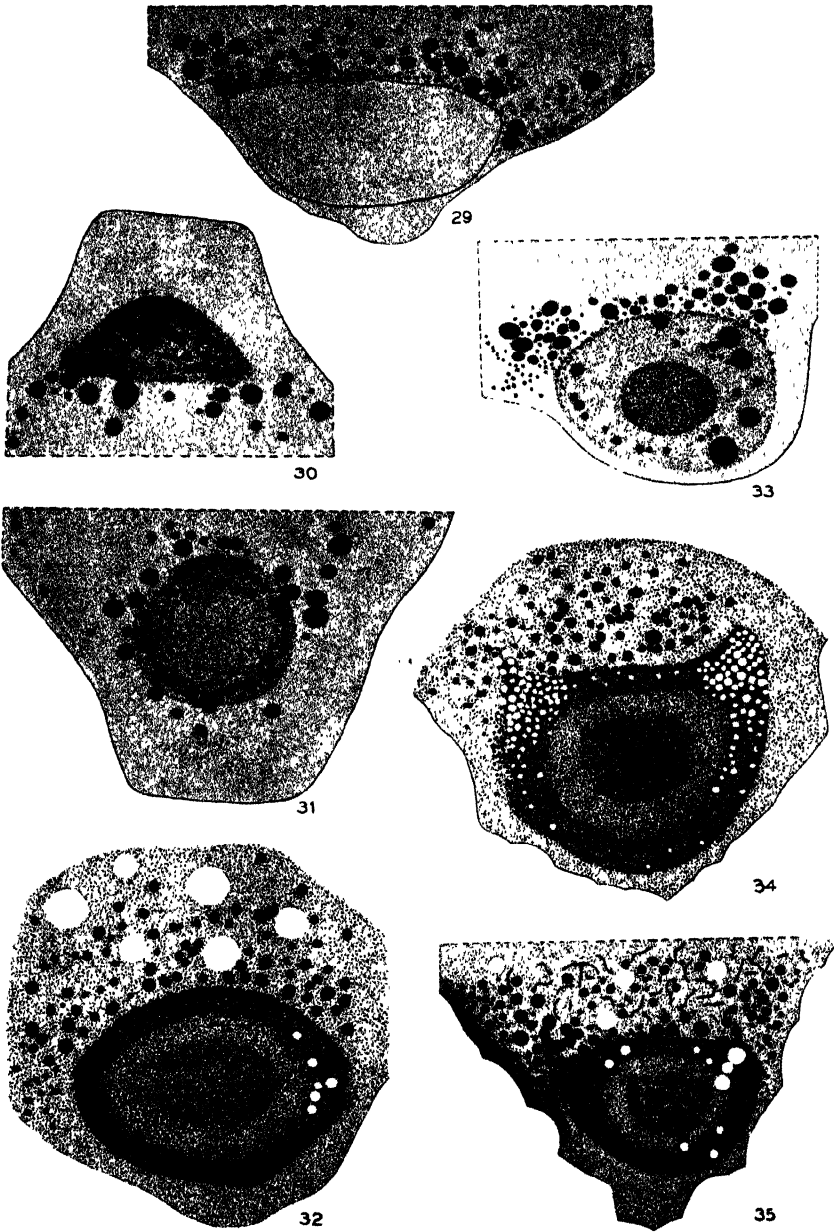


PLATE 5

EXPLANATION OF FIGURES

All figures of *Gelastorocis*; 29, 32, 33, 34, and 35 fixed in Champy, osmicated and destained with turpentine, some more than others; 30 and 31 fixed in Benda and mounted unstained.

29 Proximal end of egg showing outline of synthetic center with Golgi bodies, black and gray spheres lying in close proximity.

30 and 31 Two centers at opposite ends of same egg to show difference in shape; 30, distal; 31, proximal.

32 to 35 Proximal centers showing slight variations. Gray bodies are particularly prominent in 32, 33, and 34.

PLATE 6

EXPLANATION OF FIGURES

Figures 36 to 40, *Gelastocoris oculatus*; 41 and 42, a stone-fly. Champy fixation followed by osmic and turpentine except 39. Figure 39, Champy stained with acid fuchsin.

36 Section of egg, showing granular Golgi bodies and unequal destaining of black spheres in turpentine.

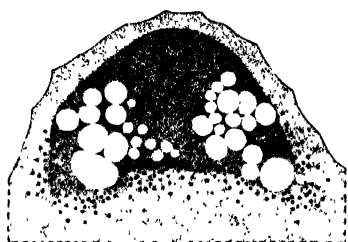
37 Distal synthetic center. Fat-spheres dissolved. Golgi bodies remain. Note shape.

38 Proximal center showing migration of fat and yolk from one edge and also streaming movements in the cytoplasm.

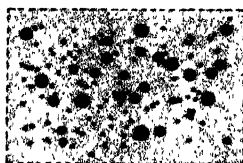
39 Rods or canals of an unknown nature.

40 Section through one side of proximal center, indicating possible transitional stages between Golgi bodies and gray bodies. Dissolved fat-spheres numerous.

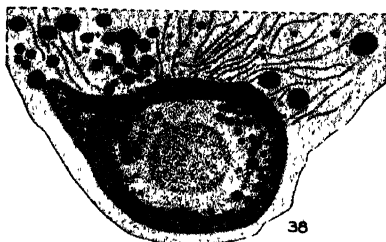
41 and 42 Eggs of stone-fly, showing distribution of yolk and fats in early development.



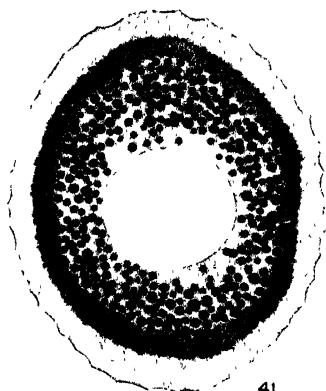
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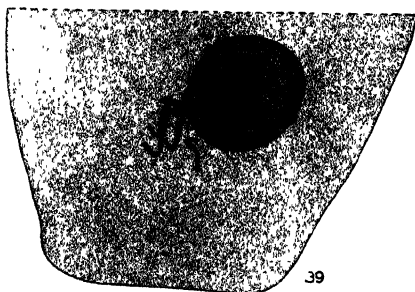
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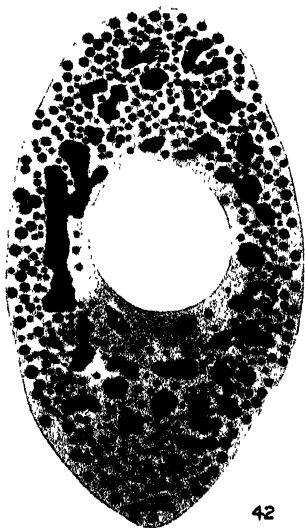
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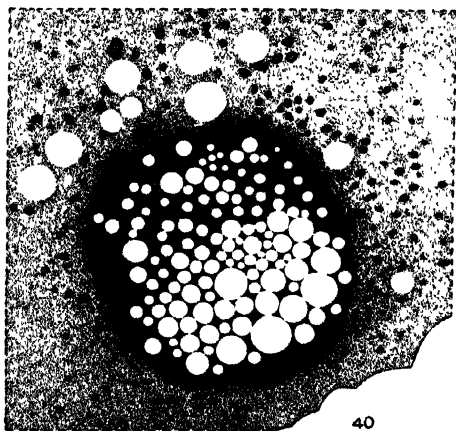
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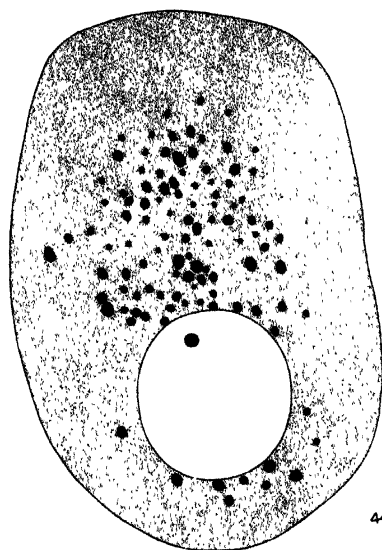
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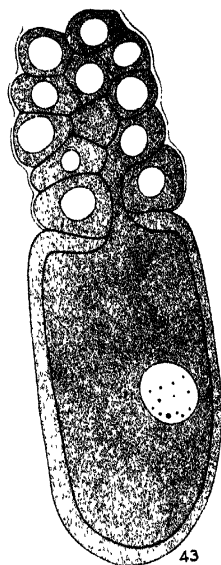
40

CYTOPLASM IN INSECT OVA
FERNANDUS PAYNE

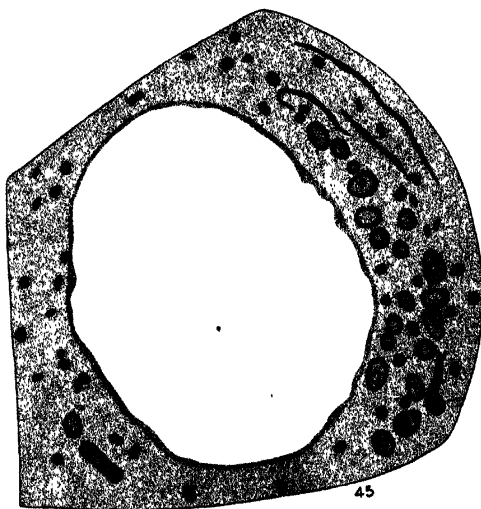
PLATE 7



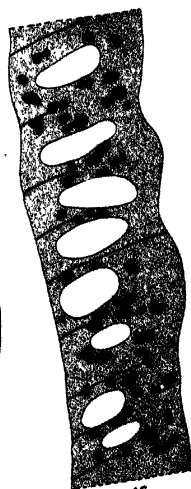
44



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PLATE 7

EXPLANATION OF FIGURES

All figures of *Cicindella sex guttata*. Fixed in Champy, followed by osmic and turpentine.

43 Lower power drawing of egg and nurse cells.

44 Low power drawing of egg, showing many bodies of unknown nature. They are the same as the large dark bodies illustrated in figures 49 and 50.

45 High power drawing of a large nurse cell showing Golgi bodies and large gray bodies.

46 Follicle cells showing Golgi bodies.

PLATE 8

EXPLANATION OF FIGURES

All figures of *Cicindella sex-guttata*. All except 52A fixed in Champy followed by osmic and turpentine; 52A, Benda, stained with haematoxylin.

47 Oogonial cell with Golgi bodies.

48 to 51 Early growth stages of eggs, illustrating Golgi bodies, the gray bodies and a third body of unknown origin, structure, and fate. Apparently the large body near the nucleus in 51 is a fusion of the large dark bodies in 49 and 50.

52A and 52B Two of the large dark bodies drawn on a larger scale, showing more details of structure.

53 Several Golgi bodies and gray bodies to show variation in size and appearance.

54 and 55 Drawn from different regions of the same egg, 54 central and 55 peripheral. Note difference in shape of Golgi bodies.

56 Section from periphery of an ovum just before yolk formation begins. Golgi bodies and gray bodies are not always distinguishable.

57 Section of periphery of egg older than 56. Yolk is present. Gray bodies are more numerous and again intergrade with Golgi bodies.

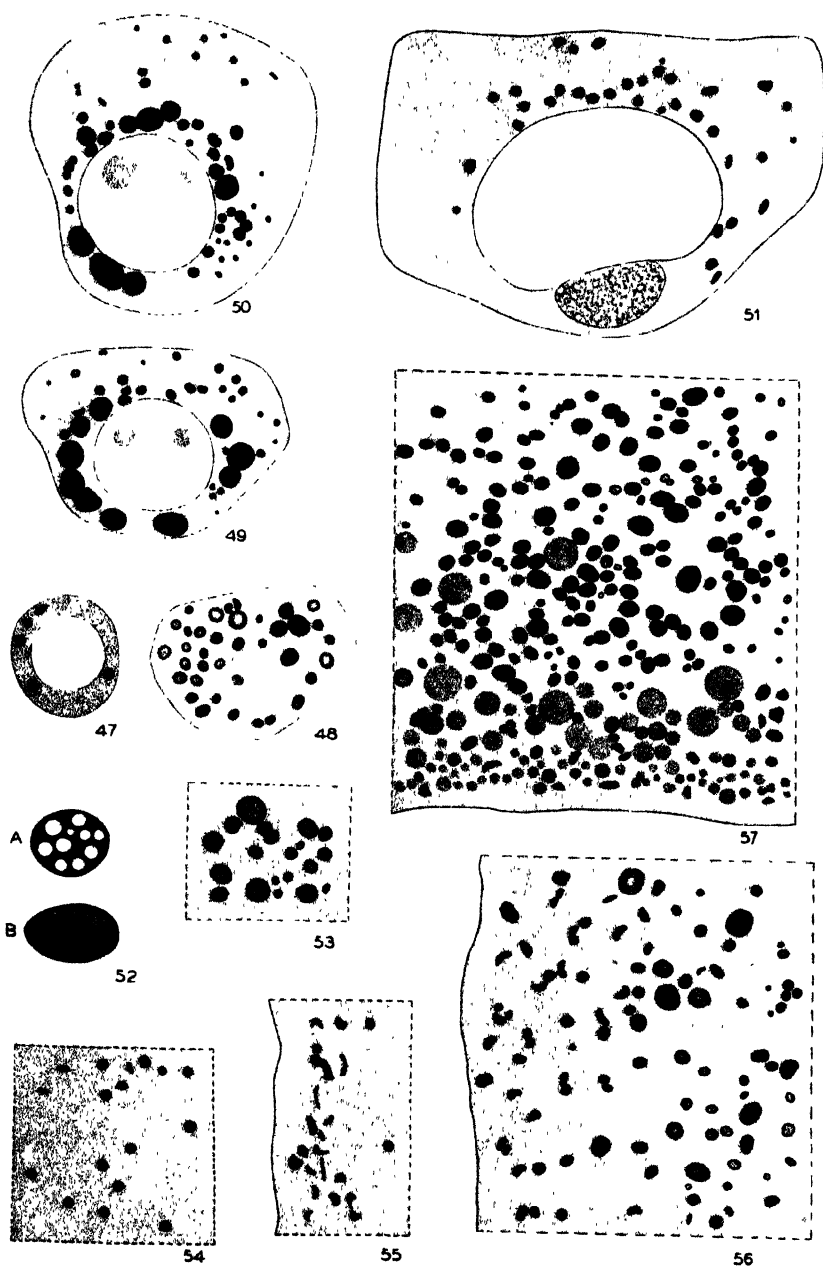


PLATE 9

EXPLANATION OF FIGURES

Figures 58 and 61, *Cicindella sex-guttata*; 62, *Cicindella repanda*. All fixed in Champy.

58 Young ovum with many chondriosomes, a few Golgi bodies and gray bodies.

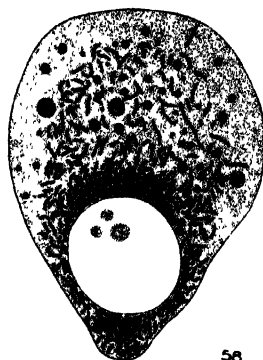
59 and 60 Two nurse cells showing transitional stages between Golgi bodies and gray bodies.

61 Ovum showing so-called vacuoles intermingled with Golgi bodies, but independent of them.

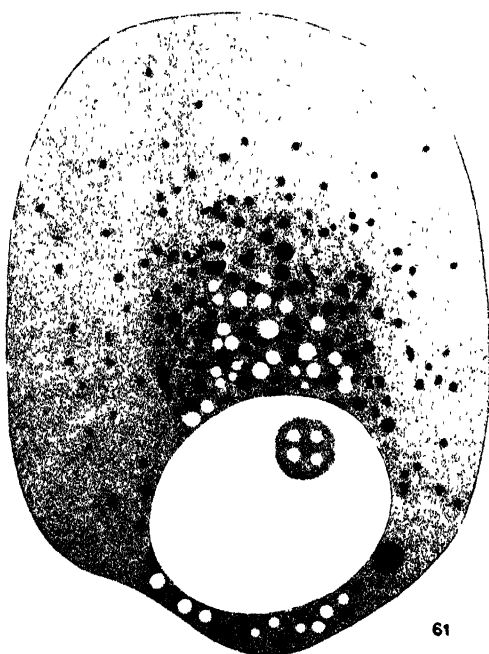
62 Part of ovum, showing vacuoles aggregated at end of egg where there are no more Golgi bodies than elsewhere. The short rods are chondriosomes. What the larger club-shaped structures are, I do not know. Note clear regions of cytoplasm near nucleus and at end of cell.



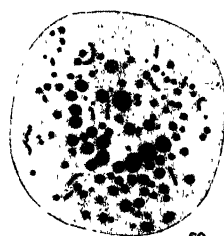
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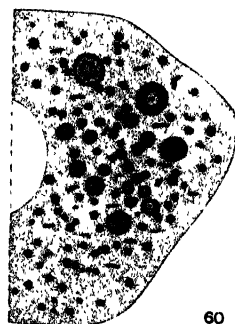
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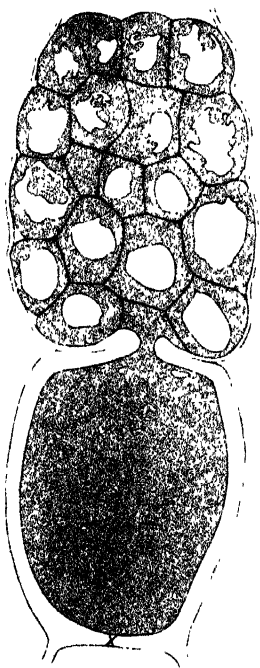
61



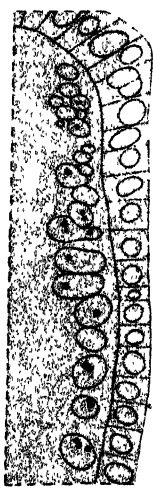
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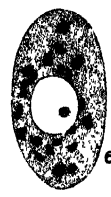
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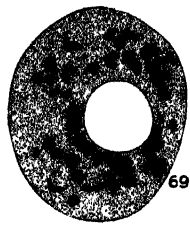
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67



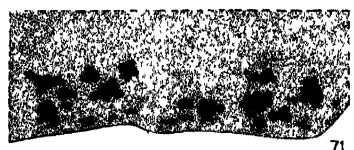
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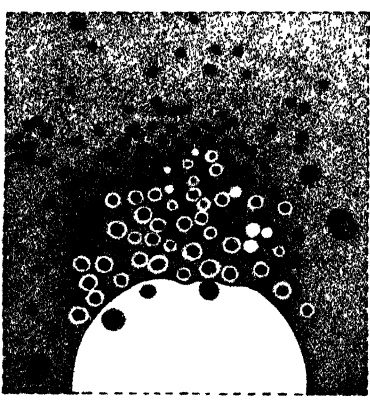
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PLATE 10

EXPLANATION OF FIGURES

63 *Cincindella sex-guttata*. Aggregate of Golgi bodies and vacuoles with yolk-like substance within them. Champy, osmic, turpentine.

64 to 66 *Colletes inaequalis* Say. Figure 64, showing ovum and nurse cells; 65, a high-power drawing from proximal end of egg, showing ovoid bodies resembling somewhat the Golgi bodies; 66, a section of the periphery of an egg, showing yolk-like granules forming inside vacuoles.

67 to 72 *Tropisternus glaber* Hbst. A series of sketches, to show formation of fatty spheres, starting with irregular masses of material in figure 68, independent of the Golgi bodies. Golgi bodies look much like chondriosomes. Champy, osmic, turpentine.

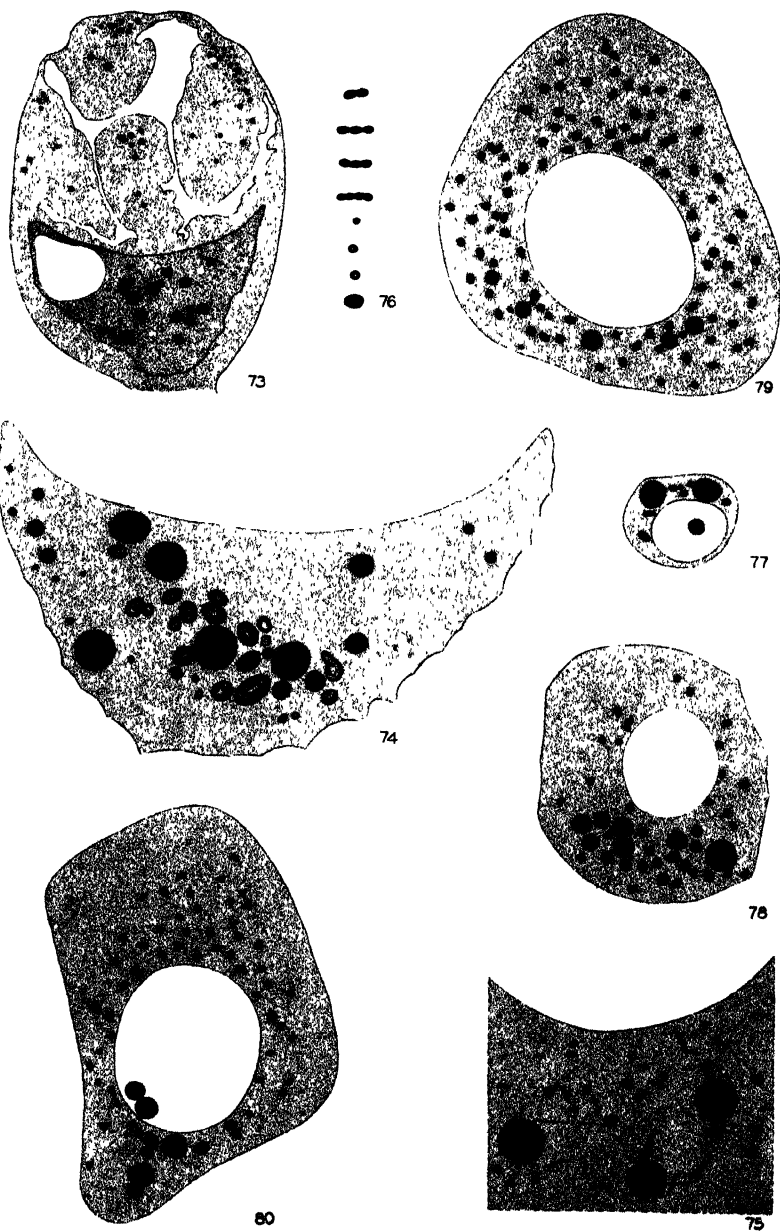


PLATE 11

EXPLANATION OF FIGURES

73 to 76 Probably *Anisolabis annulipes* (earwig). Champy, osmic, turpentine; 73, ovum and nurse cell; 74, ovum, showing gray bodies; 75, section of an egg, showing fat-spheres, chondriosomes, and vesicular Golgi bodies; 76, a series of Golgi bodies, showing variation in size and constriction.

77 to 79 *Dineutes assimilis* Aubé. Three stages in the early growth of the eggs, showing Golgi bodies and temporary fatty spheres.

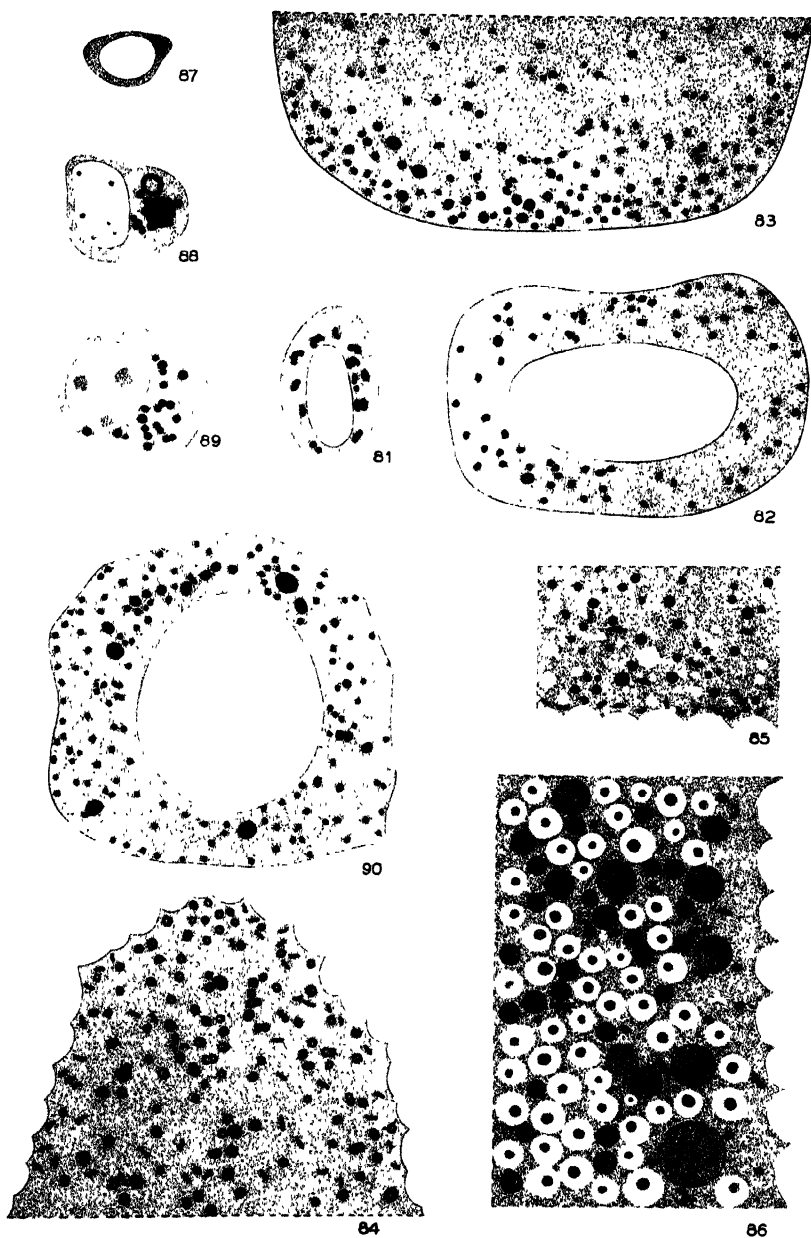
80 *Tabanus atratus*. A young ovum, showing Golgi bodies and gray bodies. Champy, osmic, turpentine.

PLATE 12

EXPLANATION OF FIGURES

81 to 87 *Oecanthus quadripunctatus*, Champy, osmic, turpentine; 81 to 84 show multiplication of Golgi bodies and their change into large vesicles; 85 shows the presence of yolk spheres and watery droplets and the absence of most of the large vesicles; 86 is a still later stage, showing fatty material within the droplets and no vesicles; 87 is an oogonial cell preceding 81.

88 to 90 *Oecanthus nigricornis*. Champy, osmic, turpentine. A series of stages in the early growth of the ovum, showing the Golgi bodies and the beginning of the formation of vesicles as in *quadripunctatus*. The vesicles become larger and blacker than in *quadripunctatus*.



DIE BILDUNG DER ERYTHROCYTEN IM KERNE DER PLASMOCYTEN; DER HÄMOGLOBINSCHWUND IM BLUTKREISLAUFE MIT DER PIGMENTMETAMORPHOSE UND OHNE DERSELBEN UND DIE BESONDERE ELIMINIERUNGSART DER CHROMATINSUBSTANZ AUS DER ZELLE IM BLUTE DES BATRACHOSEPS ATTENUATUS ESCH.

WITOLD KOMOCKI

Warschau

EINE TAFEL (MIT DREI UND ZWANZIG ABBILDUNGEN)

AUTHOR'S ABSTRACT

In the blood of *Batrachoseps* one part of the erythrocytes is formed inside the nucleus of plasmocytes as a result of the metamorphosis of chromatin substance. When erythrocytes are completely formed, they separate themselves from the plasmocytes.

Disappearance of the hemoglobin of erythrocytes with pigment metamorphosis is visible in the drawings.

When the disappearance of hemoglobin with pigment metamorphosis takes place, the color of the hemoglobin cytoplasm frequently becomes very pale. The hemoglobin cytoplasm may vanish also without pigment metamorphosis in such a way that the whole cell (nucleus and cytoplasm) swells, and the cytoplasm becomes paler, and finally only a pale shadow remains which is rather difficult to distinguish. The author has noticed in the blood of *Batrachoseps*, besides extrusion of small nuclei from the erythrocytes, also a particular way in which chromatin substance is eliminated from the cell. While the cell and the nucleus become pear-shaped, the nucleus moves toward the periphery, touching the surface of the cell with its funnel-shaped narrow side. From this narrow side the chromatin substance flows out in the form of a band stained red, according to Romanowsky.

Im Jahre 1897 erschien in Amerika in einer leider in Europa wenig verbreiteten Herausgabe eine Arbeit von G. Eisen(4) in welcher der Verfasser bewies, dass bei einer gewissen Salamanderart, nämlich bei *Batrachoseps attenuatus* Esch., der weitgrösste Teil der Erythrocyten keine Kerne besitzt, dabei sind die kernlosen, wie auch die kernhaltigen Erythrocyten von sehr verschiedener Gestalt und Grösse, nämlich von der Grösse ungefähr eines menschlichen Erythrocyt beginnend, bis zu den riesigen, z.B. auf Abbildung 21 meiner früheren Arbeit(14) vorgestellten Formen. Ferner zeigte Eisen, dass sich von den Hämatoblasten (Thrombocyten) sehr oft Protoplasmatückchen, die nachdem in sehr grosser Zahl

im Blute schwimmen, abreißen. Der grösste Teil Eisen's Arbeit ist nämlich der Beschreibung dieser Gebilde, die er als 'Plasmocyten' bezeichnete, gewidmet. Diese Beobachtungen von Eisen wurden von Giglio-Tos(8), Wright(22) und vor einigen Jahren auch von Emmel(5, 6) vollkommen bestätigt. In seiner Arbeit gab Eisen noch eine Beobachtung an, nämlich, dass er nicht selten in den Plasmocyten liegende verschiedener Grösse Erythrocyten bemerkte und erklärte sich diese Erscheinung als Resultat der Phagocytosefähigkeit der Plasmocyten. Wright, in seiner Arbeit, wo er nur sehr kurz über das Batrachosepsblut spricht, bestätigt jedoch, wie eben gesagt, Eisen's Beobachtungen und gibt eine Abbildung an, wo ein im Plasmocyt liegender Erythrocyt zu sehen ist. Emmel erwähnt in seinen Arbeiten garnichts von der Anwesenheit des Hämoglobins in den Plasmocyten.

Ich stellte oft die Anwesenheit des Hämoglobins in den Plasmocyten fest und möchte noch dabei erwähnen, dass sich dasselbe nicht nur in den frei kreisenden von den Hämatoblasten schon abgerissenen Plasmocyten befindet, sondern auch im Falle eines noch nicht stattgefundenen Abreissens (Abb. 10, 11, 12, 13); was aber die Erklärung dieser Erscheinung betrifft, bin ich mit Eisen nicht einig; ich bemerkte nämlich, dass das Hämoglobin immer innerhalb des Kernes liegt und sich wahrscheinlich infolge der Chromatinsubstanzmetamorphose bildet. Ich stellte fest, dass jeder Plasmocyt ein Kern besitzt; dieser Kern tritt aber sehr oft, jedoch nicht immer, nur in Gestalt zerstreuter, winziger Chromatinkörnchen hervor; diese Körnchen kann man nur bei der Färbung nach Romanowsky Prinzip elektiv und ganz deutlich vorstellen; es wundert deshalb nicht, dass Eisen, welcher die Präparate nach Romanowsky nicht färbte, den Kern nicht veranschaulichen konnte und die Plasmocyten als kernlose Gebilde betrachtete. Es ist nicht zu vergessen, dass Romanowsky nur mit Hilfe seiner Färbung gelungen war, die Anwesenheit des Kernes im Plasmodium malariae mit voller Deutlichkeit nachzuweisen.

Auf den Abbildungen 1 und 2 ist das Anfangsstadium der Hämoglobinbildung im Kerne dargestellt; hier ist das Hämoglobin noch sehr schwach gelb gefärbt; auch muss die Abbildung 3 als Anfangsstadium betrachtet werden; das Hämoglobin färbt sich hier gelb mit einer leicht grüner Schattierung. Auf den weiteren Abbildungen 4, 5, 6, 7, 8 färbt sich das Hämoglobin stark gelb; auf Abbildung 9 sehen wir einen schon ganz ausgebildeten Erythrocyt. Auf den Abbildungen 10, 11 und 12 ist die Hämoglobinbildung in den Kernen der vom Hämatoblast noch nicht abgerissenen Plasmocyten zu sehen: Die Abbildungen 9 und 11 weisen an, dass die schon ausgebildeten Erythrocyten eine Tendenz zeigen sich von dem Plasmocyt zu isolieren. Das wirkliche Heraustreten der Erythrocyten aus den Plasmocyten beweist der Umstand, dass manchmal im Batrachosepsblute auf der Peripherie der Erythrocyten sich Fetzen von Plasmocytensubstanz befinden. Dass das Hämoglobin sich wirklich innerhalb des Kernes bildet, ist auf den Abbildungen 1, 2, 3, 4, 5, 6, 7, 8 zu sehen; wir bemerken hier nämlich, dass die Chromatinkörner mit dickerer und dünnerer Schicht das sich innerhalb des Kernes bildende Hämoglobin bedecken. In dieser Hinsicht sind meine Abbildungen denen von Browicz und Brandts ganz ähnlich. Um noch einen Beweis dafür zu geben, dass die in den Plasmocyten sich bildende Substanz wirklich Hämoglobin ist, haben wir einige Präparate mit Benzidin nach Lepehne gefärbt; auf der Abbildung 13 befindet sich ein in dem vom Hämatoblast noch nicht losgerissenen Plasmocyte liegender, auf solche Weise gelbbräunlich gefärbter Erythrocyt.

Ich muss hier vermerken, dass alle Erythrocyten im Batrachosepsblute man in drei Gruppen teilen kann und nämlich: 1) Erythrocyten mit grossem Kerne, wie ich es auf Seite 731 meiner vorherigen Arbeit(13) darstellte, welche wie bei allen niederen Wirbeltieren, von den Hämatoblasten herkommen; 2) Erythrocyten mit kleinem Kerne, betreffs welchen Eisen vermutete, dass es ein im Erythrocyt liegender Parasit sei, und, 3) kernlose Erythrocyten.

Im Jahre 1897 entdeckte Browicz(1, 2) bei seinen Leberstudien die Anwesenheit des Hämoglobins in den Leberzellen des Hundes, er bemerkte dabei, dass dieses sich nicht nur im Protoplasma, sondern auch im Kerne befindet; der Verfasser war sehr umsichtig und vermied jede Vermutung hinsichtlich der Art der Hämoglobinerscheinung in den Leberzellen, ohne seine Beobachtung mit der Phagocytose zu erklären; seine Arbeit illustrierte er mit vielen ganz überzeugenden Abbildungen. Im Jahre 1909 bestätigte Brandts(3) die Beobachtungen von Browicz über die Hämoglobinanwesenheit in den Kernen, entdeckte dabei das Hämoglobin bei den Hunden nicht nur in den Kernen der Leberzellen, sondern auch in den Nierenzellen. Diese eingehende Arbeit wurde auf vier Tafeln mit auch sehr überzeugenden Abbildungen illustriert. Dass manchmal der im Plasmocyt geformter Erythrocyt einen Kern (Eisen's angeblichen Parasit), wie es Eisen auf seiner Abbildung 79 zeigte, besitzen kann, wundert schon nicht mehr, nachdem es festgestellt wurde, dass der Erythrocyt sich im Kerne bildet; es ist sehr wahrscheinlich, dass manchmal ein Teil der Kernsubstanz unaufgebraucht in dem gebildeten Hämoglobin bleibt. Unsere Abbildungen 9 und 11 unterscheiden sich von den anderen, wo der Bildungsprozess des Hämoglobins dargestellt ist, dadurch, dass neben den, wie es scheint schon gänzlich gebildeten Erythrocyten, sich noch ein Teil von unaufgebrauchter Chromatinsubstanz befindet.

Ich muss hier noch vermerken, dass Knoll(10, 11), Villa(21), und Ferrari(7) vermuten, das Hämoglobin entsteht im Kerne und nämlich deshalb, weil die Spalten zwischen den Chromatinfäden sich acidophil färben und auch darum, weil der unmittelbar mit dem Kerne grenzende Protoplasmateil sich manchmal viel intensiver, als das übrige Protoplasma färbt.

Prenant(16) entdeckte das Hämoglobin im Kerne mit Hilfe der Benzidinreaktion.

Retterer(18) sucht das Entstehen der Erythrocyten der Säugetiere aus den Zellkernen zu beweisen, dabei sollte das Protoplasma schwinden und der ganze Kern sich in einen kernlosen Erythrocyt umwandeln.

Bei meinen Untersuchungen über das Blut der Schildkröte (12) traf ich einige pigmentierte Erythrocyten; da eine grössere Zahl solcher Zellen, ungeachtet der langdauernden Untersuchungen, nicht zu finden war, so stellte ich nur aus Pflicht des Referenten zwei solche Zellen auf der Tafel (l.c. Abb. 58 und 59) vor. Im Batrachosepsblute finden sich ähnliche Gebilde ziemlich oft; es zeigte sich dabei, dass es unbestreitbar zerfallende mit Pigmentbildung Erythrocyten sind.

Auf Abbildung 14 ist eine Zelle mit sichtbarem, aber sich beinahe garnicht färbendem Protoplasma zu sehen; kleiner viereckiger Kern mit gelockerter Substanz färbt sich hellviolett; aus der Abbildung ist zu sehen, dass dieser Kern schwindet; beinahe von allen Seiten ist er mit breiter Zone bräunlicher oder grünlichbräunlicher Pigmentkörner umgeben. Auf Abbildung 15 bemerken wir drei aneinander liegende kernlose Erythrocyten, von welchen zwei ganz normale sich gut färbende sind, der dritte jedoch färbt sich ungewöhnlich schwach; auf bedeutendem Teil seiner Peripherie ist ein Streifen zu sehen—es scheint, als ob dies eine Membran des Erythrocyt wäre; zwischen diesen Streifen und der Erythrocytensubstanz befindet sich ein breiter mit eben-solchen, wie auf Abbildung 14 dargestellten Körnchen, gefüllter Raum. Auf Abbildung 16 sieht man einen sich sehr schwach färbenden von allen Seiten mit Streifen aus eben-solchen Körnchen umgebenen Erythrocyt. Auf Abbildung 17 ist ein runder, sich sehr gut färbender Erythrocyt dargestellt; von allen Seiten ist er mit einer dunklen fast schwarzen Membran umringt; dieselbe verlängert sich auf gewisser Stelle in einen birnenförmigen, mit entweder ganz schwarzen Körnchen oder schwarzer sich zusammenfliessender Masse gefüllten Sack. Die den Erythrocyt umgebene Membran enthält auch auf gewisser Stelle ebensolche Körner, die Membran selbst färbt sich aber hier schwächer. Auf Abbildung 18 ist eine Gruppe von sieben sich sehr schwach färbenden Erythrocyten dargestellt; der Raum zwischen denselben, wie auch um die ganze Gruppe herum liegender breiter

Streifen ist mit einer entweder schwarzen, bräunlichen, gräulichen oder blauen Pigmentkörnermasse ausgefüllt. Es unterliegt keinem Zweifel, dass wir hier mit der Pigmentbildung aus den zerfallenden Erythrocyten zu tun haben.

Wie weit es uns bekannt ist, wurde solcher im Blutkreislaufe stattfindender Prozess bis jetzt nur äusserst selten beobachtet; so gibt List(15) in seiner vorläufigen Mitteilung an, dass er den Pigmentzerfall der Erythrocyten in den Gefässen der Oberhaut bei Triton beobachtet hat. Was den Erythrocytenzerfall in den inneren Organen betrifft, ist hierbei Reich's Arbeit(17) zu erwähnen; der Verfasser entdeckte in der Milz der Kröte Blutkörperchen, die auf Pigmentkörner zerfielen; schliesslich ist das Hämoglobin gänzlich zerfallen und es ist nur ein sich sehr schwach färbender und verkleinerter Kern geblieben.

Der auch im Blutkreislaufe statfindende Prozess, nämlich das Schwinden des Erythrocytenhämoglobins durch Auflösung, habe ich schon im Blute der *Salamandra maculosa* in einer von meinen Arbeiten(13) gezeigt; denselben Prozess habe ich auch im Blute des *Batrachoseps* gesehen. Auf Abbildungen 19 und 20 sind zwei grosse, als ob aufgequellte kernhaltige Erythrocyten, deren Hämoglobinprotoplasma sich sehr schwach färbt, dargestellt; auf Abbildung 19 ist das Protoplasma kaum zu sehen. Solche sehr schwache Färbung des Hämoglobins bemerkt man oft im *Batrachoseps*blute auch in den kernlosen Erythrocyten—es bleiben nämlich nur ihre Schatten zurück.

Die Frage über den Zerfall der Erythrocyten bei pathologischen Bedingungen, z.B., unter dem Einfluss verschiedener hämolitischen Giften, ist im gewissen Grade aufgeklärt; in welcher Weise dagegen ein Teil der Erythrocyten bei normaler Bluterneuerung schwindet—ist die Frage bis jetzt noch ganz dunkel; es ist deshalb kein Wunder, dass Peyton Rous(19) seine Monographie unter anderen mit solchen Worten endet: "The reader who has come thus far will not fail to have noted that much of current theory upon blood destruction attests to the theorist's abhorrence of a vacuum."

Im Batrachosepsblute habe ich das Heraustreten der kleinen Kernen (Eisen's angeblichen Parasiten) aus den Erythrocyten bemerkt, wie auch das Heraustreten bei den Hämatoblasten der Chromatinkörner aus dem Kerne ins Protoplasma, um weiter Plasmocyten zu bilden. In diesem Blute beobachtete ich aber noch eine besondere Eliminationsart der Chromatinsubstanz aus der Zelle; nämlich die ganze Zelle nebst dem Kerne nimmt eine birnenförmige Gestalt an, der Kern schiebt sich zur Peripherie der Zelle und grenzt mit seiner trichterförmigen Verengung mit der Oberfläche der Zelle oder ragt das Trichterende sogar aus der Zelle heraus; aus dieser nämlich verengten Stelle fliesst das in Form kürzeres oder längeres Bandes purpurrot gefärbte Chromatinsubstanz aus. Auf Abbildung 21 ist gerade ein solcher Erythrocyt mit einem sehr pyknotischen Kerne, aus welchem sich die Chromatinsubstanz in Form eines sehr langen, auf ganzem Gesichtsfelde sichtbaren Bandes ausgiesst, dargestellt. Auf Abbildung 22 haben wir mit einem Erythrocyt, dessen Hämoglobin polychromatophil gefärbt ist, zu tun; die Kernsubstanz ist ziemlich gelockert, der sich ausgiesende Chromatinstreifen ist viel kürzer, als in der vorherigen Zelle. Auf Abbildung 23 ist ein Hämatoblast mit sehr pyknotischem Kerne, der mit einem engen Protoplasma-streifen, nicht auf der ganzen Peripherie, bedeckt ist, dargestellt; auch hier ist ein kurzes und enges aus ziemlich dicken Chromatinkörnern zusammengesetztes und purpurrot gefärbtes Band, das aus dem Kerne austritt, zu sehen; rechts von dieser Zelle bemerken wir einige wahrscheinlich von diesem Bande während des Blutausstreichens abgerissene Chromatinkörner. Es ist hier zu vermerken, dass man solche Ejakulation der Chromatinsubstanz im Batrachosepsblute nur selten beobachten kann. Ob dieses Chromatinausgiesen bis zum Heraustreten aus der Zelle des ganzen Kernes führt, oder sich der Prozess unterbricht und der Kern mit vermindertem Chromatinquantum weiter in der Zelle bleibt—ist die Frage vorläufig noch nicht zu entscheiden. Auf Abbildung 22 haben wir mit einem jungen Erythrocyt, dessen

Protoplasma noch schwach mit Hämoglobin gefüllt ist und sich polychromatophil färbt, zu tun. Die Zelle Nr. 21 muss man auch als junge betrachten, da, wie es aus der Abbildung zu sehen ist, das Hämoglobin sich bräunlich-gelb, anstatt gelb-rosa, wie wir es gewöhnlich in gänzlich gebildeten, reifen Erythrocyten sehen, färbt. Der Umstand, dass wir hier mit jungen Zellen, deren Entwicklungszeitlauf noch nicht beendet ist, zu tun haben, spricht wahrscheinlich dafür, dass nur ein Teil des Chromatins aus dem Kerne austritt.

Die Elimination der Kerne aus den Erythroblasten beim Menschen und bei den Säugetieren ist seit Rindfleisch eine bekannte, nur von einigen Autoren bestreitbare Tatsache. Wie es scheint, gehört das Heraustreten der Chromatinteilchen und sogar ganzer Kerne aus der Zelle bei den Protozoa nicht zu sehr seltenen Erscheinungen; R. Hertwig (9) beschreibt, z.B., das Kernheraustreten bei *Actinosphaerium Eichhorni*; Schaudinn (20) berichtet über denselben, von ihm in den lebenden Zellen beobachtenden Prozess bei der *Entamoeba coli* Lösch und bei seiner *Entamoeba histolitica*.

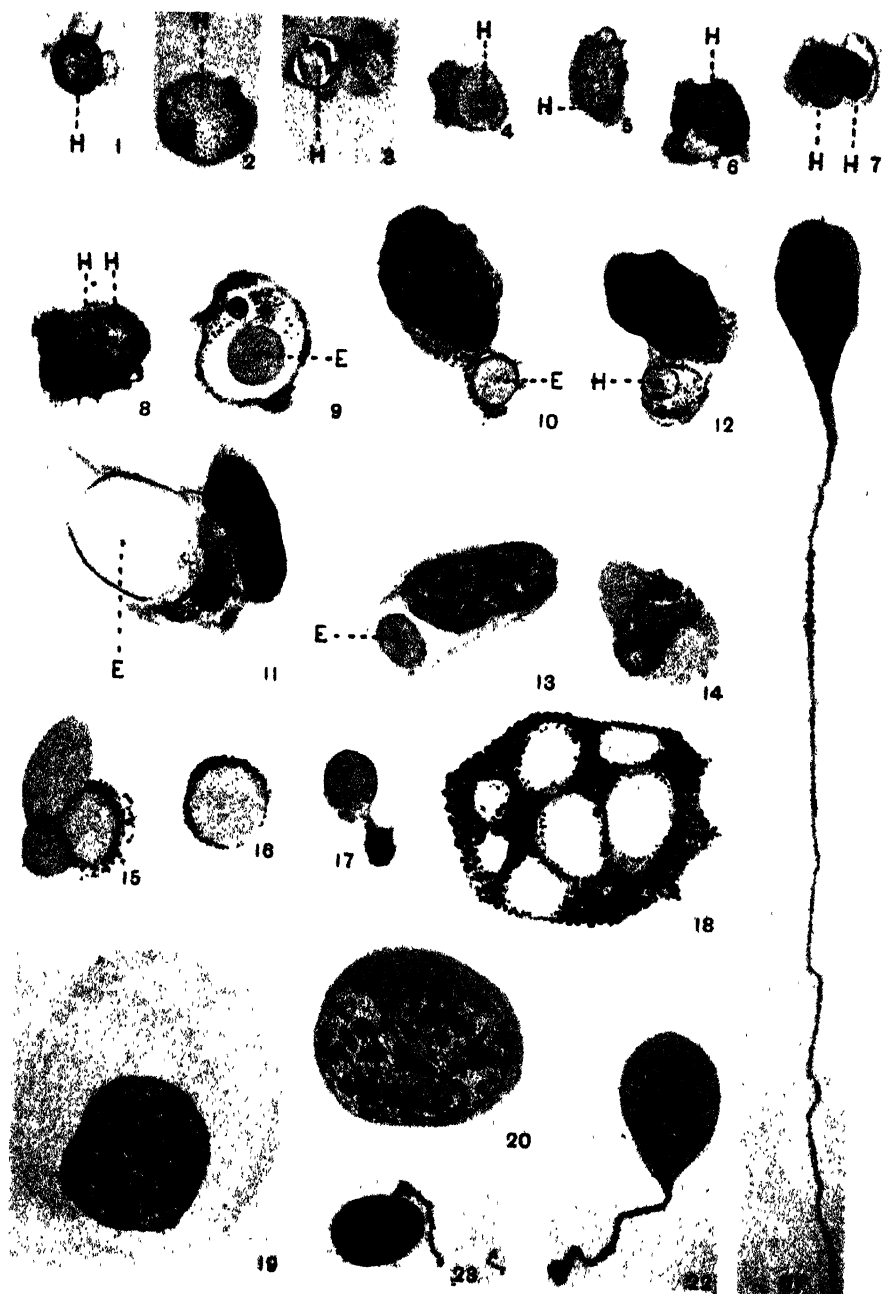
Die Abbildungen wurden bei Oc. 4, Obj. Hom. Im. 1/12 Reichert von den Ausstrichpräparaten, welche mir auf mein Ersuchen in sehr grosser Zahl weiland V. E. Emmel, Professor der Anatomie an der Universität Chicago, kurz vor seinem Tode in äusserst freundlicher Weise zugesandt hatte, ausgeführt. Ein Teil von den Präparaten wurde von ihm nach Romanowsky-Wright, ein anderer—vom mir nach Romanowsky-Pappenheim gefärbt.

Auf den Abbildungen 1, 2, 3, 4, 5, 6, 7, 8, und 12 zeigt lit. H den Ort der Hämoglobinprotoplasmaabildung innerhalb des Kernes. Zum besseren Verständniss dieser meinen Abbildungen bitte ich den Leser, die schönen farbigen Abbildungen in den Arbeiten von Browicz und Brandts durchzumustern. Auf den Abbildungen 9, 10, 11, und 13 befinden sich unter lit. E schon gut geformte Erythrocyten.

ERWÄHNTE ARBEITEN

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